Highlights

1.- Prebiotic definition could change in the future with the aim to broad the concept

2.- Protein engineering tools are useful to make novel enzymes with glycosidase activity

3.- Seaweeds and marine microalgae are promising sources for prebiotic oligosaccharides

4.- Future research will focus on well-designed prebiotic dietary interventions

5.- Research needed on underlying mechanisms of action and on human intervention studies

*Manuscript

Current state and latest advances in the concept, production and functionality of prebiotic oligosaccharides

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Abstract

The increasing knowledge on the complexity of the gut microbiota and its interaction with the host is challenging the concept and definition of prebiotics. This review emphasizes the recent advances in the enzymatic production of prebiotic oligosaccharides, the use of renewable and/or alternative sources, i.e. seaweeds and marine microalgae, and the clinical evidences towards the beneficial effects of prebiotic oligosaccharides in the gut and other systems. It also focuses on the current gaps and research needs in the field of prebiotics, highlighting the necessity to gain a deeper knowledge on mechanisms that govern the interactions between prebiotics and human gut microbiota, on chemical structure-function relationship of prebiotics or to develop well-controlled human intervention studies to support claims of health benefits.

Evolution of the prebiotic concept.

Although since the 50´s is known that selected carbohydrates stimulate the growth of beneficial bacteria in the gut [1], their use for the production of health promoting functional foods began to be subject of interest many years later, when intensive research on the role of intestinal microbiota in many aspects of health highlighted the potential importance of those carbohydrates, generally referred to as prebiotics.

The prebiotic concept has evolved with the state of scientific knowledge. It was first defined in 1995 as “a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health” [2*]. The development of metagenomics approaches and other “omic” tools have significantly improved our knowledge on the complexity of the gut microbiota and its interaction with the host, so that, the original concept of prebiotics has been revised many times over the years [3].

The mechanisms responsible for changes in the gut microbiota due to consumption of prebiotics remain to be fully clarified but the information available on intestinal microbiota indicates that cross-feeding occurs so, for instance, acetate and lactate produced by Bifidobacteria and Lactobacilli can be converted to butyrate by other species that can be beneficial to health. This indicates that the impact of prebiotics on gut microbiota composition and activity may be broader than previously thought, revealing that one of the main criteria traditionally used for the classification of prebiotics, that is selectivity, is currently challenged. In consequence, the need for a new definition of prebiotics has been recently expressed with the aim of shifting the focus towards ecological and functional features of the microbiota more likely to be relevant for host physiology, such as ecosystem diversity, the support of broad consortia of microorganisms and production of short chain fatty acids (SCFAs) [4**]. Indeed, the assumption that a richer microbiota is also more healthy [5] has led to the proposal of the inclusion of ecological biodiversity in the definition of prebiotics [6]. In addition, the current knowledge on the gut microbiota composition and metabolic activity makes unclear and too simplistic the differentiation between beneficial and detrimental bacterial species. Therefore, it is very likely that the concept of prebiotic will be redefined in the future with the aim to broad the concept. Nevertheless, a deeper knowledge of the mechanisms that govern the interactions between prebiotics and human gut microbiota will be necessary before a definitive definition of prebiotics can be reached.

Current and future trends in production of prebiotic oligosaccharides.

The food industry needs more efficient, sustainable, simple, and less expensive processes for their application on a large scale. The production of prebiotic oligosaccharides can be hindered by their structure complexity and related costs that may render non-competitive industrial production. Prebiotic oligosaccharides can be found naturally in foods or, alternatively, they can be produced by enzymatic or chemical synthesis from disaccharides or other substrates, as well as by hydrolysis of polysaccharides. Recognised prebiotics, such as lactulose, galactooligosaccharides (GOS), fructooligosaccharides (FOS) and inulin, are good examples but besides them there are a variety of potential prebiotics that are obtained from natural sources or synthesized enzymatically. A considerable number of carbohydrates varying in monosaccharides composition and order, configuration and position of glycosidic linkages have been proposed as potential prebiotics, most of them based on the utilization of inexpensive and abundant agro-industrial waste such as pectin-derived oligosaccharides or xylooligosaccharides (XOS). Lactose obtained from cheese whey permeate and sucrose are also efficient sources to produce by enzymatic synthesis lactose-derived trisaccharides, including 4-galactosyl-kojibiose, lactulosucrose [7] or lactosucrose [8]. The main carbohydrates present in tofu whey permeate as sucrose, raffinose and stachyose are enzymatically converted to mixtures of fructosylated α-galactosides and FOS [9]. The hydrolysis and subsequent transgalactosylation of lactulose to produce novel prebiotic oligosaccharides has been described by using β-galactosidase from different microbial sources [10].

Although it has yet to be improved, the hydrolysis of polysaccharides is normally the most reliable choice for oligosaccharide production on a large scale, due to its reproducibility and high yield. Oligofructose can be obtained through selective hydrolysis of inulin by action of endoinulinase [11]. In the case of fungal inulinases, several studies of cloning and modification have been made to achieve great efficiency, obtaining yield up to 91.3% of oligofructose with degrees of polymerization (DP) mainly between 3 and 6 [12]. In contrast, the synthesis of FOS by transfructosylation of sucrose using a modified fructosyltransferase produced oligomers with similar DP and yield of 61% [13*]. During the hydrolysis of polysaccharides, it is important to avoid the release of monosaccharides. Thus, Rajagopalan *et al.* [14] efficiently produced XOS from hardwood xylan using an immobilized endoxylanase of *Clostridium* strain BOH3, and only xylobiose and xylotriose were released.

The design of enzymes with specific features aimed at new acceptor substrates, to better control regioselectivity and/or to increase the reaction yield contributes to enhance the glycodiversification and quality of the attained products [15, 16]. Protein engineering approaches have led to major achievements to create novel enzymes [17], and to improve their performance (i.e., productivity, activity, and pH and thermostability) [18]. Nevertheless, protein engineering currently requires significant research efforts in each case because the structure-function relationship of many enzymes with glycosidase activity is poorly understood due to the paucity of studies on their three-dimensional structure determination [19].

Finally, the production of oligosaccharides by a fermentation process using genetically modified microorganisms can be an emerging strategy for the industrial manufacturing of oligosaccharides, as it has been reported for 2'fucosyllactose [20].

Seaweeds and marine microalgae are alternative sources for the finding of potential prebiotic candidates.

Over the last years, the spectrum of prebiotics has not been restricted to the recognised oligosaccharides and the use of conventional agro-food sources. Seaweeds and marine microalgae are one of the most important sources of polysaccharides or oligosaccharides resultants thereof that are not decomposed by the enzymes of the upper part of gastrointestinal tract with unique biochemical and fermenting characteristics.

Laminarans, fucoidans, floridean starch, sulfated galactans, xylans, mannans, glucomannans, pectins and alginic acid are some of the main structures present in marine algae [21, 22]. These polysaccharides can be transformed to oligosaccharides by means of different methods. Ultrasound has been used for xylan, carrageenan and agarose, whereas microwave for exopolysaccharides. Oligomer formation by free radical performed with Cu^{2+} or Fe^{2+} and H_2O_2 has been utilised for fucoidans. Hydrolysis by phosphoric acid is the best selection when uronic acids are the main components of the polysaccharide, while thermal-acidic hydrolysis with diluted HCl has been used for agar and agarose-derived oligosaccharides. In general, physical techniques may present lower or not side effects, for instance, both ultrasound and microwaves are not toxic and very effective from the point of view of energy and time consuming [23].

Due to the large groups of algae, species and strains of the same genus, these compounds are complex and heterogeneous molecules whose structural elucidation is difficult, the structure-function establishment being a huge challenge. Thus, the biological properties normally result from a complex interaction of several structural features, including the sulphation level, distribution of sulphate groups along the polysaccharide backbone, molecular weight, sugar residue composition and stereochemistry. A wide range of bioactive properties have been already attributed to polysaccharides and oligosaccharides algae derivatives such as virucidal, antibacterial, antifungal, antiinflammatory, immunomodulatory, anticoagulant or antithrombotic, antiproliferative, tumour suppressor, apoptotic, antilipidemic, hypoglycaemic, hypotensive, antiaging and antioxidant activities. In addition, other properties such as antinociceptive acting as a peripheral analgesic agent and neovascularization and hepatoprotective effects have also been described [24].

Algae-derived oligosaccharides have been proposed as potential prebiotic candidates, although the fulfilment of the criteria still has to be shown for most of them [23]. The controversial results obtained by various authors, even when the same seaweed species are considered, may result from the heterogeneity of the chemical composition, differences in the structure and molecular weight of polysaccharides, the age and habitat of algae, etc. Algal polysaccharides have a great potential for emergent prebiotics to be used directly as dried biomass, or as nutraceuticals, after isolation from the biomass or from the culture medium. They may be incorporated in foods, or administered as medicines. The advance of enzyme knowledge together with the finding of new enzymes (bioprospecting) from marine bacteria and molluscs should allow altering polysaccharide structures and producing novel prebiotics [23].

Clinical evidences towards beneficial effects of prebiotic oligosaccharides.

During the last few years has significantly increased our knowledge about the diversity of human microbiota composition, its relation to health and its interaction with the diet. Currently, there is growing evidence indicating that our gut microbiota is deeply implicated in a wide range of metabolic functions extending beyond the gut and prebiotics may play an important role in this complex system. In fact, the development and progression of certain human diseases can be associated to a dysbiosis of gut microbiota [25]. Nowadays, different therapeutic strategies can help modifying the negative effects provoked by the gut microbiota imbalance. One of the most used to modulate composition and metabolic activity of gut microbiota is focused on dietary interventions using prebiotics [26**]. Indeed, they are becoming key components of a health-promoting diet given their success in the attenuation of many diseases and improvement of health at distant sites [27*].

It is well established the physiological effect of prebiotics in the human colon through the production of SCFAs, particularly acetate, propionate, and butyrate typically at a ratio of 3:1:1 [28], as well as other products that lower the local pH, stimulate mucin production by colonocytes, induce production of immunomodulatory cytokines and promoting ammonia and amine excretion [29**].

Among SCFAs, butyrate is the most important gut metabolite in host-microbiome interactions as it exerts potent effects on a variety of colonic mucosal functions such as inhibition of inflammation and carcinogenesis, antiproliferative properties, induction of apoptosis, reinforcing components of the colonic defence barrier and decreasing oxidative stress [30**]. Therefore, the investigations are oriented towards the search of new prebiotics that stimulate not only the growth of *Bifidobacterium* and *Lactobacillus* but also other beneficial bacterial species in the human colon such as butyrate producers, as it is the case of the *Roseburia/Eubacterium rectale* group [31]. Some other postulated bacteria targets associated with health are: i) *Akkermansia muciniphila*, a mucin-degrading bacterium that resides in the mucus layer and is present at lower levels in obese and type 2 diabetic subjects [32]; however, levels of this bacteria were found to be higher in patients with colorectal cancer than in healthy controls [33]; ii) *Faecalibacterium prausnitzii*, since reduced numbers are present in infectious colitis and Crohn's disease patients [34]; iii) others as *Ruminococcus bromii*, *Oxalobacter formigenes* or different species of Bacteroides [35*]. Nevertheless, more research is needed on the underlying mechanisms of action and on targeted intervention studies, since host- microbiome interactions cannot be explained by the effect of single factors [36].

Recent reviews on the topic of the health benefits of prebiotics describe studies on many other physiological effects of prebiotics, including those associated with bone density and strength, hepatic encephalopathy, immune function, cardiovascular and dermatological health, metabolic diseases as well as on neurobiological changes associated with prebiotic intake [27*, 37**]. Concretely, the gut-brain axis is being particularly explored in the recent years because it has been pointed out that the gut microbiota regulates central nervous system homeostasis through immune, vagal and metabolic pathways [38]. Abnormalities in the composition of the gut microbiota have been associated to several neuropsychiatric disorders, such as autism [39], schizophrenia and major depressive disorder [40].

Despite the positive effects showed in animal studies, data in human studies are rather scarce, so, further studies will be necessary to clarify the role of prebiotics in human health and clinical nutrition. The mostly studied prebiotics in animal and model trials are complex mixtures of oligo- and polysaccharides such as FOS (inulin and oligofructose) and GOS which are termed as nutraceutical ingredients due to the available data [28]. However, only a small number of studies have investigated the effect of monosaccharide composition and type of glycosidic linkage on the metabolism of prebiotics by microbiota [41-46]. Lastly, there are currently very few reliable data on the digestibility of candidate prebiotic molecules as this is a very challenging property to determine and requires large quantities of carbohydrates [47].

Future trends

Figure 1 illustrates the research needs and potential areas of exploration in the field of prebiotics in the coming years. All proposed prebiotics include a broad diversity of structures but the current state of knowledge about relationship between the molecular structure and the effects of prebiotic compounds on microbiota and its metabolites is scarce. Increasing knowledge on this topic it is expected to contribute to the synthesis of new tailored prebiotics with enhanced target-specific functional properties. Isolation and characterization of pure prebiotic carbohydrates with different monosaccharide composition, DP and type of glycosidic linkage, together with the study of the fermentation pathways of such oligosaccharides at the molecular level will increase our knowledge on chemical structure-function relationship of prebiotics and to some extent, in the not too distant future, to the more rational design of carbohydrates targeted at particular species of probiotic microorganisms for personalized therapies. Another worthwhile direction for future research would be to study the potential combined effect of two or more prebiotics in order to stimulate biodiversity of the human gut microbiome.

While preclinical studies in animal models have provided evidence of the beneficial contribution of prebiotics in many aspects of health, the scarce data on human intervention studies warrant further well-controlled human trials to support claims of health benefits of prebiotics. In addition, it is necessary to established what outcomes (biomarkers) have to be measured, as well as to identify fundamental and timely methodology which could make easier the comparison among different studies. These considerations could shed light on the unravelled mechanistic link between microbial activity and potential health benefits and to define what constitutes a healthy gut microbiota. However, this is a challenging task due to the complexity of the human gut microbiome, whose composition can be influenced by different aspects such as: host genetics and state (e.g., fiber may adversely affects the symptoms of people with irritable bowel syndrome), as well as environmental and lifestyle (dietary habits) factors [48**, 49].

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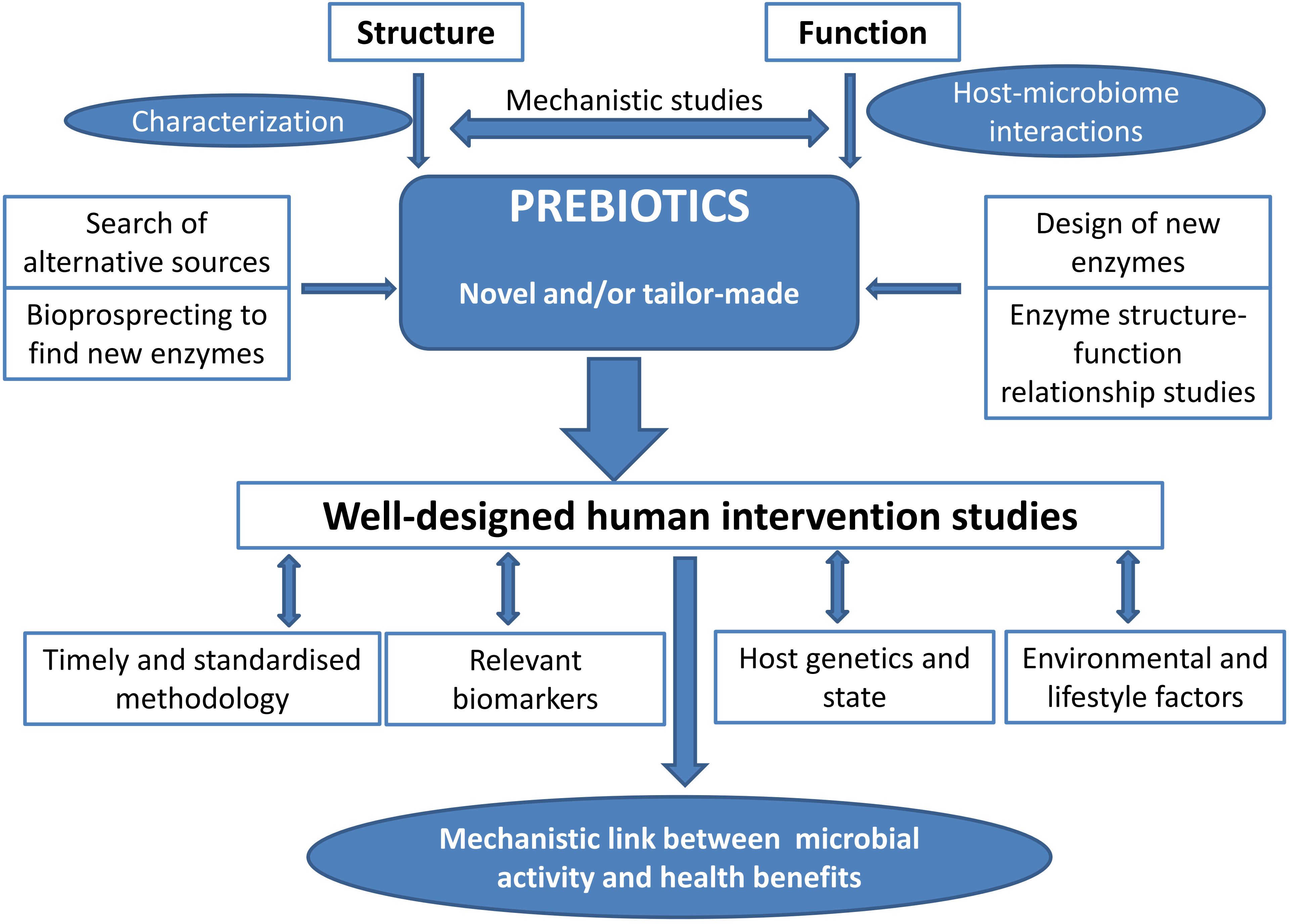
Figure legends.

Figure 1. Research needs and future areas of exploration in the field of prebiotics.

Figure


Structure
Function
Characterization
Mechanistic studies
Host-microbiome interactions
PREBIOTICS
Novel and/or tailor-made
Search of alternative sources
Bioprosprecting to find new enzymes
Design of new enzymes
Enzyme structure-function relationship studies
Well-designed human intervention studies
Timely and standardised methodology
Relevant biomarkers
Host genetics and state
Environmental and lifestyle factors
Mechanistic link between microbial activity and health benefits