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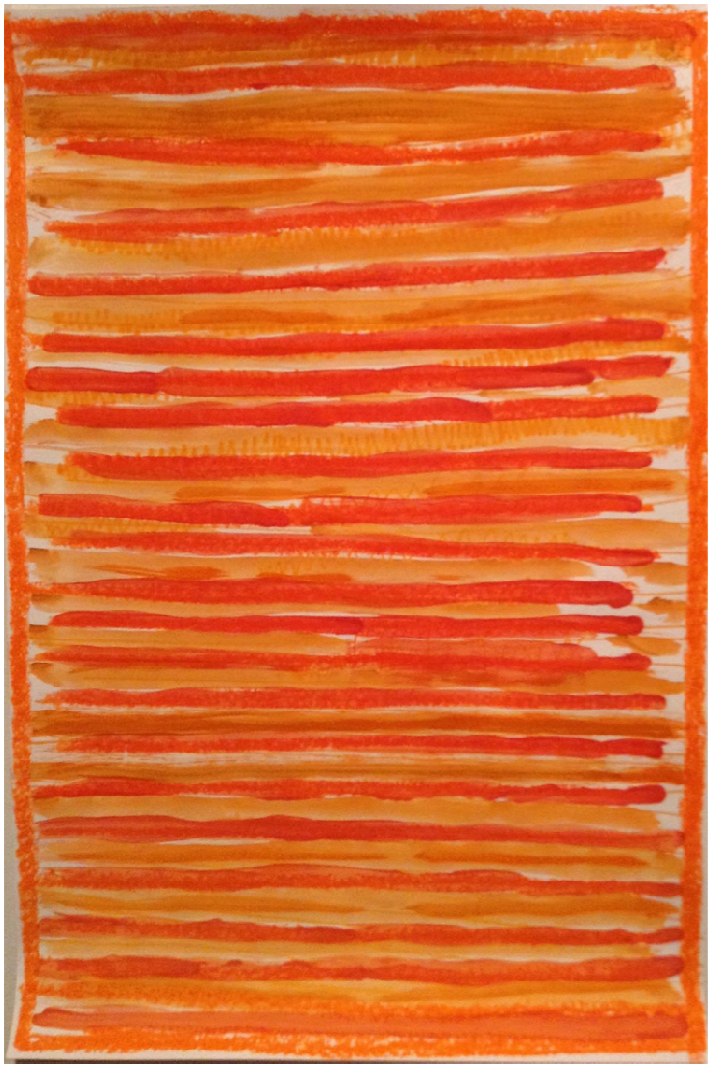

Master Thesis

"Probiotics and Prebiotics in Human Health and Animal Welfare"

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Athens, September 2016

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For my Mentor,

Effie Tsakalidou,

my Teachers

K.Papadimitriou, E. Drosinos,

S.Alexandraki and M.Kazou,

my familyand friends

to whom i'm grateful for

their contribution,

support,

words of wisdom

and encouragement.

Abstract in Greek (Περίληψη)

Στη παρούσα μελέτη σκοπός ήτανη ανασκόπηση των τελευταίων ερευνών και της βιβλιογραφίας σχετικά με τους μικροοργανισμούς που φέρουν προβιοτικές ιδιότητες και τών υδατάνθρακων που χαρακτηρίζονται ως πρεβιοτικά, με στόχο τη κατανόηση, τη σύντομη ανάλυση της λειτουργικότητας, του ρόλου, της συμβολής και των εφαρμογών των παραπάνω ως προς την συμβολή τους στην ομοιόσταση, στην θωράκιση και στη διατήρηση της υγείας. Η δομή της αυτής της εργασίας χωρίζεται σε τρία κύρια κεφάλαια: το πρώτο είναι ο πρόλογος, το δεύτερο αναφέρεται στην εφαρμογή των προβιοτικών και πρεβιοτικώνως προς τον άνθρωπο και το τρίτο ως προς την εφαρμογή τουςστα μονογαστρικά και πολυγαστρικά οικόσιτα ζώα, τα βρώσιμα ψάρια, τις μέλισσες καθώς και για τα κατοικίδια ζώα. Πιο συγκεκριμένα,στην πρώτη ενότητα κάθε κεφαλαίου, παρουσιάζεται μια σύντομη εξιστόρηση, την εξέλιξη του ορισμού των προβιοτικών, που ακολουθείται από τις ιδιότητές τους, όπως καθορίζονται από τις κατευθυντήριες γραμμές του FAO/WHO για τον άνθρωπο και του FEEDAP της EFSA όσον αφορά τα ζώα, που χαρακτηρίζουν ένα στέλεχος προβιοτικό, οι μηχανισμοί δράσης τους στο ξενιστή και κύρια παραδείγματα των ευεργετικών δράσεωντους σε νόσους, ασθένειες και παθήσεις, στην ικανότητα πρόληψης αλλά και θεραπείας αυτών. Αναλύεται η ισχύουσα νομοθεσία για την Ευρώπη, τις Ηνωμένες Πολιτείες και την Ιαπωνία, τα οικονομικά δεδομένα και οι σύγχρονες τάσεις στην εφαρμογή τους. Εν συνεχεία, με τους ίδιους άξονες γίνεται μια ανασκόπηση στη δράση των πρεβιοτικών που σχετίζονται με την προώθηση της υγείας, σύμφωνα με την κατευθυντήρια γραμμή των FAO/AGNs.Εν κατακλήδη, ύστερα από διάφορες μακροχρόνιες μελέτες και μετα-αναλύσεις, φαίνεται πως υπάρχουν αρκετοί ισχυρισμοί και παγκόσμιοι οργανισμοί που επιβεβαιώνουν μέσα από σειρές μελετών ότι οι προβιοτικοί μικροοργανισμοί διαδραματίζουν σημαντικό ρόλο για την υγεία του ανθρώπου και των ζώων, ρυθμίζοντας, ενισχύοντας και προάγοντας την υγεία, ενώ σε συνδιασμό με την τήρηση μιας ολιστικής διατροφή που περιέχει πρεβιοτικά, ιδίως αυτά που μπορούν να χαρακτηριστούν ως μη διαλυτές φυτικές ίνες, φαίνεται να προάγεται αυτή η οφέλημη δράση. Η εργασία αυτή αποτελεί σύνοψη σειράς μελετών, ερευνών εν αρμονία με την ισχύουσα νομοθεσία, για τα προβιοτικά βακτήρια και των πρεβιοτικών υδατανθράκων, ως συμπληρώματα διατροφής, εν δυνάμει φαρμακευτικά προϊόντα και πρόσθετες ύλες σε ζωοτροφές, των οποίων η εφαρμογή για τα τελευταία έχει τεθεί σε ισχύ.

Abstract

Over time, living organisms have coevolved hosting a variable community of microorganisms, which provide a range of biochemical functions that are needed and cannot actually be provided without them to the host.From latest studies and analysis of bibliography is revealed that the properties and functions of probiotic bacteria and prebiotic carbohydrates, are critical connected with the enhancement and maintenance of homeostasis and health. For many years, microbial adjuncts have been used to supplement the diet of farm animals and humans. They have evolved since the 1990s to become known as probiotics, i.e. functional food with health benefits. A defined group of carbohydrates has been proved to enhance the gastrointestinal microbial diversity, nutrient absorption and digestibility in a beneficial way for the host organism. The structure of the present review is separated in three main chapters: the first is the prologue, the second is referred to probiotics and prebiotics for humans and the third of the above in animals (livestock, aquatic animals, pollinators and pets).As so, in the first section of each chapter, it is presented a brief analysis of probiotic's history and the evolvement of their definition followed by their properties, as established by the guidelines of FAO/WHO for humans and EFSA's FEEDAP panel as regards to animals, mechanisms of action and examples of positive effects on health and welfare. Economic facts and current trends are presented, as well. Additionally, legislation in Europe, Japan and the United States of America is discussed. Last but not least, future perspectives beyond the probiotic framework, are pointed.After the above, in the second section of each chapter, the above sub-sections are being presented in the same order for prebiotics. Furthermore, regarding the prebiotic properties, a putative guideline of FAO/AGNS is presented and discussed. Studies suggest that diet and nutrition can shape the microbiota. As so, a healthy, holistic, diet that includes prebiotic componenments especially with fermentable substances, is proven to be beneficial as growth substances for the probiotic associates. After long-term studies, there are several claims from both independent scientists and worldwide organizations confirming through a series of studies that pro- and prebiotics play an important role in humans and animals in enhancing, regulating and promoting health.

Field of study: Food technology and safety, nutrition

Key words:

Probiotics, prebiotics

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Chapter 1: Prologue

We are placed in a world full of microorganisms. These are not only around but inside and outside all living organisms.Over time, mammals and plants have coevolved to host a complex community of microorganisms, which provide a range of biochemical functions that are needed and cannot actually be provided without them. In the years followed there wasn't paid so much attention as now when coming to microbes, until a decade ago when it was unraveled that the total collection of genes found in all the microbes associated with a particular host, or the so-called microbiome, was linked to health while having a key role in behavior, especially in mammals.

As far as humans are concerned and with the Human Genome Project being completed in 2003, microorganisms are nowadays regarded as an extra functional organ, since the total count of procariotic cells is almost equal with the count of eucariotic cells in the human body. Since then, research groups around the world identifyed and characterizied the microbiota in both healthy and diseased humans and concluded that there are distinct taxa across different areas of the human body and huge variation at lower taxonomic levels among individuals .

From the beneficial microbial community of mammals,several microorganisms with specific properties and modes of action have been isolated, identifiedand many of them have been classified as probiotics. Probiotics are defined as ''live microorganisms which confer a beneficial act to the host'' and can be found among every living organism as a part of the microflora. There is a worldwide effort to find potential probiotics by isolating those strains from animal, plant and food product origins.

Probiotic strains include mainly genera of lactic acid bacteria (LAB), propionic acid bacteria (PAB), and bifidobacteria. Several strains that fall into the probiotic spectrahave been used for the preservation of milk and vegetables for centuries. Currently, a wide variety of probiotic strains are industrially used as starter cultures, cocultures, or bioprotective cultures to improve preservation, flavors, and texture of milk, vegetables, meat, and cereal products. Moreover, depending on the strain or more accurately on its mechanisms of action observed, a probiotic treatment can be administrated for prevention, induction, maintenance of remission and relapse prevention inseveraldiseases and disordersfor both humans and animals, as it will be presented and discussed(FAO/WHO 2001; FAO/WHO 2002).

Prebiotics are mainly charbohydrates extracted from plant origins, but they can be synthesized aswell.Due to their physicochemical and organoleptic properties, they are used as food treatment agents/additives for centuries. On top of that, prebiotics areaddressedin theFood and Agriculture Organization of the United Nations (FAO) and Food Quality and Standards Service (AGNS) technical meeting reportas ''non-viable food components that confer a health benefit on the host associated with modulation of the microbiota''(FAO/AGNS 2007). On that account, several prebiotics when consumed in proper amounts seem to enhance the action of probiotic strains in the mammalian body.

For many years, microbial adjuncts have been used to supplement the diet of farm animals and humans. They have evolved since the 1990s to become known as probiotics, i.e.

functional food with health benefits(Bernardeau & Vernoux 2013). As so, in the first chapter the probiotic and prebiotic properties and mechanisms of action are analysed as proposed in the guidelines established by FAO/WHO. In the second chapter the above are being presented in the sameorder in a relation to the enhancement of health and animal welfare. Furthermore, regarding the positive effects of probiotics, only examples of single strains are presented and discussed.Current legislation for pro- and prebiotics is discussedand the regulatory bodies in specific countries, sincethey may be categorized differently. Economic facts and current trends are presented as well.

Chapter 2: Probiotics and Prebiotics for Humans

2.1. Section I: Probiotics for Humans

2.1.1 Definition of probiotics

'Probiotic' is a word derived from Latin and Greek language meaning "pro-bios" thus "for-life" and was first used in 1954 (Ferlazzo *et al.,*2011; Morelli & Capurso, 2012; Binns, 2013). The definition of probiotics has been reformulated throughout the years and there is still an ongoing debate for it within the scientific community. In 1954, Werner Kollath described probiotics as "active substances that are essential for healthy development of life"(Guarner *et al.,* 2005). In 1965, Lilly and Stillwell described probiotics as "substances secreted by one microorganism that stimulate the growth rate of another"(FAO/WHO, 2001), in addition with Parker who in 1974 defined probiotics as 'organisms and substances which contribute to intestinalmicrobial balance'. This definition related probiotic use to the intestinal microflora but the inclusion of substance gave it a wide subtext which would include antibiotics(Schrezenmeit & De Vrese, 2001).

Later on, Fuller (1989) wanted to emphasize on the microbial nature of probiotics. He redefined the word as "dietary supplement of live microbes that beneficially affect the host by improving its intestinal balance"(FAO/WHO, 2001). A similar definition was given by Havenaar and Huis Int Veld (1992), who however redefined probiotics as "live cultures, consisting of one or more microbes which, when administered to animals or humans, are beneficially affecting the host by improving the properties of the intestinal flora"(Schrezenmeit *et al.*, 2001).A few years later, in 1998, probiotics have been described byGuarner and Shaafsma as "live microorganisms, which when consumed in sufficient quantities, produce beneficial effects on the host beyond those of basic nutrition" (FAO/WHO, 2001). Moreover, in the next year, ILSI (International Life Sciences Institute) Europe Working Group defined it as "A live microbial food ingredient that is beneficiall to health "(ILSI 1999). Thereafter, the definition proposed by Schrezenmeit and de Vrese (2001) sets out that "probiotics are a preparation of microorganisms or a product that contains live, defined microorganisms, which positively alter the composition of the microflora by implantation or colonization in a host's apartment, after repeated periodical reintroduction, thus exerting beneficial effects on health". In 2001, scientists on behalf of the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) debated on the emerging field of probiotic microorganisms for the first time. An agreement on the appropriate definition of the probiotic as"live microorganisms which when administered in adequate amounts confer a health benefit on the host" came out, hence adopted and accepted worldwide.

In 2013, the non-profit organization International Scientific Association for Probiotics and Prebiotics (ISAPP), including members of theFAO/WHO Expert Panel and Working Group as well, organized a meeting of clinical and scientific experts with an interest in the re-examination of the concept of probiotics with clearer guidelines for defining all aspects of the probiotic field. Grammatical correction on the definition of the term came out and worded about the same, as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host'. Generally, the probiotic definition refers to a wide spectrum of applications that must be microbial, viable and beneficial to hosts' health (ISAPP, 2014)or (Sanders *et al*. 2014).

2.1.2 History of probiotic microorganisms

The probiotic research field has developed in the past few decades explosively, starting from a few observations and publications by individual researchers about 100 years ago.

Evidence from wall paintings dating back to 2500 BC. reveal that Sumarians had the habit of inoculating milk to induce fermentation (Ezema, 2013). Further historical evidence have been documented as well, like the one during the Genghis Khan era (12th century), when Mongolians had observed the health benefits of fermented milk consumption and ritualistic sprayed it on horses and riders as a form of protection (Butel, 2013). Independent observer may be, among others, Hippocrates (460-370 BC.), who said that 'all diseases begin in the gut' and who was the first to correlate health promotion with food consumption, claiming that 'food should be your medicine and your medicine should be your food'(C.R Soccol *et al.*, 2013). Other documentations are found in Old Testament (Genesis 18:8) where Abraham who owes his longevity to sour milk consumption indicating that probablythe first foodsmade containing living microorganisms were fermented milk(Schrezenmeit *et al.,* 2001).

Theodor Escherlich in 1886 researched the benefits of lactic acid bacteria and lactose in feedand to the physiology of the infant's digestion system(Patterson & Burkholder, 2003). In 1892 Albert Doderlein, a gynecologist, proposed that vaginal infections may be treated with lactobacilli strains. According to him, vaginal bacteria are lactic acid producers, which through the metabolism of sugars are suppressing the growth of pathogens(Lamont *et al.,* 2011).

Beyond those observationsElie Metchnikoffis considered by many the godfather of the probiotic concept. After observing Bulgarian longevity peasants for a while,in 1907 he published the book "The Prolongation of Life" translatedin English and related long term health benefits with fermented dairy foods consumption, like yoghurt and butter milk while summarizing what was known at that time of the Bacteriology of fermented milks (Kroger, Kurmann & Rasic, 1992;Ezema, 2013). Moreover, he proposed that gut bacteria may play a role in aging and in adverse health problems, since the gut contains proteolytic microorganisms which are slowly intoxicating human bodies but those may be displaced when 'useful microbes' are being consumedlike the 'Bulgarian bacillus' as he named it(FAO/WHO, 2001;World Gastroenterology Organisation Global Guideline, 2011).

At the same period (1906), alongside Metchnikoff, Henry Tissier, a Frence pediatrician who maybe heard of the incident, analyzed the microbiota of stool samples from a breastfeed healthy infant and infants who suffered from diarrhea. He observed that Y-shaped bacteria were remarkably more in the samples of the healthy subject. After performing some tests he named that Y-shaped stain "*Bacillus bifidus communis"*(FAO/WHO 2001; WGO, 2011). After a while he proposed at the Biological Society the administration of a drink to accelerate the building up of the gut flora, with cultures of *Bacillus acidparalactici* or a mix of the cultures *B.acidiparalactici* with *B. bifidus*, for diarrhea prevention in children (Butel, 2013).

Another scientist who during WWI found a great differentiation between healthy and non-healthy subjects by analyzing stool samples for gastrointestinal Shingellosis treatments, was Alfred Nissle. During a serious incident of Shigellosis outbreak, he analyzed the stool microbiota from a healthy non-infected soldier and isolated the strain *E.coli*, or "*E.coli* Nissile 1917" as he proposed to be named, and administrated the specific culture for treatment(WGO Global Guideline, 2011). The first probiotic dairy drink was produced by Yakult Honsta Company in 1935, which is in the markets till today. Minoru Shirota in 1930 was the first who successfully isolated and cultured a *Lactobacillus* strain capable of surviving in the gastrointestinal conditions. He named that strain *Lactobacillus casei* strain Shirota and used it as a starter for the Yakult fermented milk production(Tamime *et.al.*, 2005).

In the years passed, the field has grown tremendously, since each year there are more publications and more people being interested in this field, thus more information is coming out.

2.1.3 Probiotic Properties

Nowadays after long-term studies it is known and widely accepted that probiotics are live microorganisms, generally bacteria but also yeasts, that when ingested alive in sufficient amounts have a positive effect on the health going beyond the nutritional intakes as they may operate through a nutritional and/or health and/or sanitary effect.In 2002, a FAO/WHO Working Group followed up, and provided a list of useful guidelines regarding the characterization and evaluation of potential probiotics.

In 2001, the joint committee of WHOandFAO recommened a generallized scheme regarding the criteria for the evaluation of the probiotic potential in microorganisms and substaining a coherent and principled approach concerning health claims. Furthermore, in 2002, the joint committee of WHO and FAO specified the 2001 guidelines of probiotic evaluation, since the isolation of the strain can be from different natural ecological niches, including the host organism ment to be administrated or from acidic food products(FAO/WHO, 2002).

As regards genetically modified microorganisms (GMM), those are far from being applied in functional foods, at least in the European legal frame(Lee & Salaminien, 2009).

The following Figure 1,as adopted from the FAO/WHO document of 2002, represents the minimum requirements needed for a probiotic status to be made.

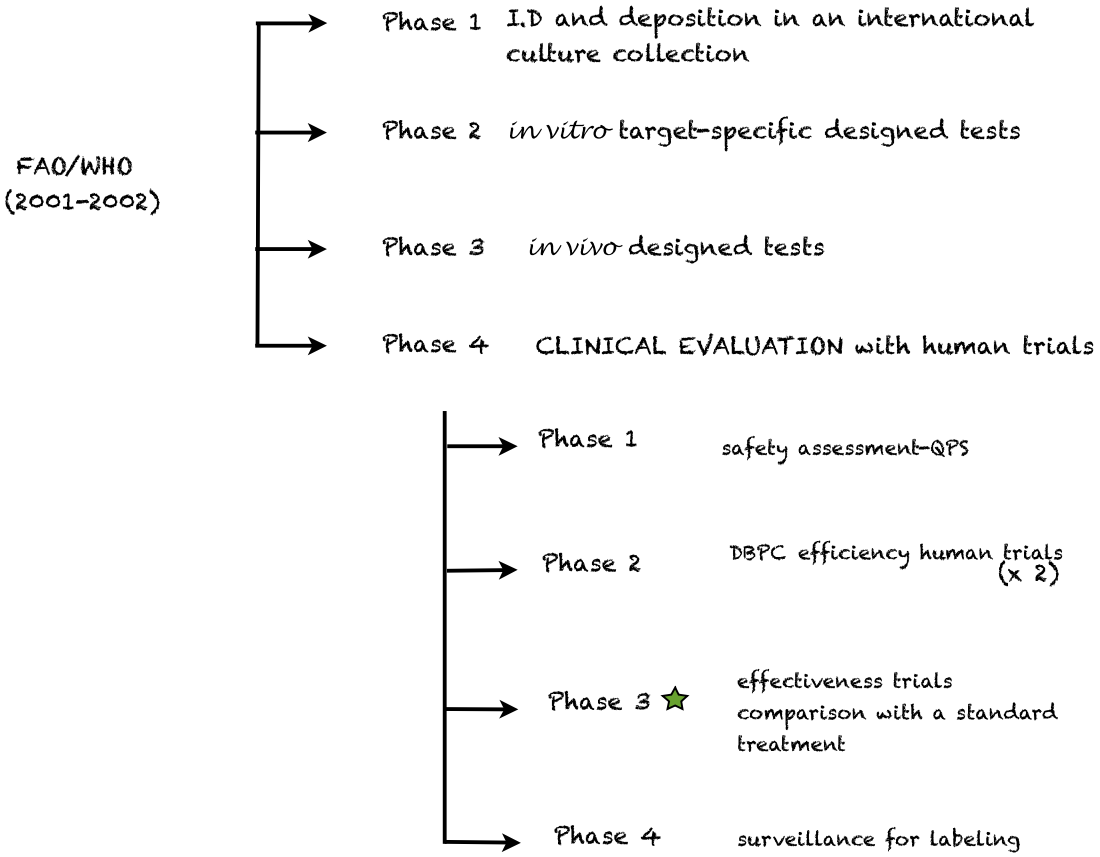

Figure 1. FAO/WHO recommendated evaluation process of 2002.
Reccomended phases for the evaluation of a potential probiotic strain for human administration.In the second phase of the fourth, the double-blind-placebo controlled human trials may be perfomed again as a second independent evaluation process. The third phase of the phase 4 is not required for probiotics delivered in food products or when used as starters for fermended food products

Further analysis of each phase is presented below;

1st Phase studies

According to this scheme, the 1st phase of those guidelines refers to the identification (I.D) of the strain and the deposition of it in an international culture collection(Morelli & Capurso, 2012). Since the probiotic effects are mainly strain specific,it is crutial to identify the microorganismdown to species level (genetic typing)(Morelli & Capurso, 2012). Strain typing has to be performed with a reproducible genetic method, or by using a unique phenotypic trait or with a combination of those two(FAO/WHO, 2002). It is recommended that phenotypic tests take place first, followed by genetic identification, using such methods as DNA/DNA hybridization, 16S RNA sequencing, pulse field gel electrophoresis (PFGE),RDP (ribosomal data base project) for confirmation of identity or other internationally recognized methods(FAO/WHO, 2001, Vaughan, Amor & De Vos, 2007,Lee & Salaminien, 2009).

Determination of the presence of extrachromosomal genetic elements, such as plasmids, can contribute to strain typing and further characterization (FAO/WHO, 2002).After following those actions, the profiles of the 'unknown' strains can be, after comparison to a pattern database of known species, identified down to strain level, in order to determine whether the new probioticcandidate belongs to one of the established, named taxa, as well as to enable accurate surveillance and epidemiological studies (FAO/WHO, 2002). Exception on strain I.D prosses, are in cases where there held suitable scientific substantiation of health benefits that are not strain specific (FAO/WHO, 2002).

Nomenclature is the assignment of the given names to the taxonomic groups according to specified rules (FAO/WHO, 2001). The Consultation endorses that probiotics must be named according to The International Code of Nomenclature of Bacteriafor ensuring understanding internationally(FAO/WHO, 2001; Morelli & Capurso, 2012)as per the International Committee on Systematics of Prokaryotes (ICPS) (available at http://www.the-icsp.org/).The names are Latin or Latinized Greek derivations, often descriptive of some key property of the organism, and are printed in italics. By classifying microorganisms into groups and naming them genetically, including theirsource of isolation,metabolism and behaviour in specific conditions, cell morphology, pathogenesis, evolutionary relationships and by other unique properties of the strain (Binns2013), it is possible to communicate effectively in aspects of a particular organism (FAO/WHO, 2001).For example those two websites provide listings of valid, approved bacterial names: List of Prokaryotic Names with Standing in Nomenclature (available at http://www.bacterio.cict.fr), and Bacterial Nomenclature Up-to-Date (available at http://www.dsmz.de/bactnom/bactname.htm). Afterwards it is recommended that all strains be deposited in an internationally recognized culture collection (FAO/WHO, 2001; Morelli & Capurso, 2012).

2nd Phase studies

The 2nd phase includes target specific designed *in vitro*tests in which a screening of their survival and functionality in specific mammalian body conditions is evaluated. The survival in the stressful gastrointestignal (GIT) conditions (low pH and high bile salts concentrations), the ability to transitory colonize the GIT, which is related with the adhesion to mucus and/or intestinal epithelium, as well as the antimicrobial activity through the production of antimicrobial molecules or the ability to inhibit/displace the adhesion of pathogens are the most commonly employed criteria for screening potential probiotic strains(FAO/WHO, 2002).The recommended trials according toFAO/WHO Working Group Report (2002) are:

- Resistance to gastric and bile acidity
- Adherence to mucus and/or human epithelial cells and cell lines
- Antimicrobial activity against potentially pathogenic bacteria

- Ability to reduce pathogen adhesion to surfaces
- Bile salt hydrolase activity
- Resistance to spermicides (applicable to probiotics for vaginal use)

However all of these tests require validation with in vivo trials(FAO/WHO, 2001;2002). Further analysis of each trial is discussed above;

Resistance to gastric and bile acidity

For the screening of putative probiotic bacteria most works simulate the GIT conditions *in vitro (*Papadimitriou *et al.,* 2015). For example, after mastication the first barrier that bacteria must overcome is the low pH values of the stomach with values ranging from 1 to 3 and mean exposure times of 90 min(Tsakalidou *et.al.,* 2005, Lee & Salaminien 2009). Into the duodenum in which bile is secreted, the pH value rises to 6–6.5, but bile salts are poured from the gallbladder to reach concentrations ranging from 1.5 to 2% during the first hour of digestion and decreasing afterwards to 0.3% w/v or lower (Noriega *et al.,* 2004; Lee & Salaminien, 2009). The residence period, in the small intestine, until 50% of emptying its fluids, takes between 2.5 and 3 h and the transit through the colon could, take up to 40 h (Camillieri *et al.,* 1989; Lee & Salaminien, 2009). There the pH values are close to neutral (from 5.5 to 7) and the physiological concentration of bile salts is lower. Therefore, the source of gastric juice and especially the source of the sample of bile (bovine, porcine, or human) can modify the tolerance pattern of the strains (Dunne *et al.,* 1999; Lee & Salaminien, 2009,Papadimitriou *et al.,* 2015). Those limitations can be overcomeif*in silico* analysis take place first (Papadimitriou *et al.,* 2015).

Adherence to mucus,cell lines and human epithelial cells

The intestinal mucosa is covered by a layer of different types of epithelial cells, which are distinctly different in the different regions of GIT, and is in contact with the lumen, the space inside the intestignal tube (Lee & Salaminien, 2009). The intestinal epithelium isconsisted bysecretory and absorption epithelial cells which are almost completely covered by a protective mucus gel composed predominantly of mucin, glycoproteins acting as the anatomical GIT site, in which the host first encounters gut bacteria (Deplancke *et al.,* 2001; Lee & Salaminien, 2009, Papadimitriou *et al.,* 2015).This structure is the most important part of the intestignal immune system for protecting hosts' system from invasion, and it is collectively refered as GALT (gut-associated lymphoid tissue) (Müller *et al.,* 2005, Lee & Salaminien, 2009).

Several models have been employed to screen the ability of putative probiotic strains to adhere to the intestinal epithelium cells (IEC). In general, studies have often been carried out with cellular lines obtained from human colon adenocarcinomas such as Caco-2 (ATCC HTB-37), HT-29 (ATCC HTB-38) and HT-29MTX, with the last ones being able to produce mucin (Muller *et al.*, 2009; Papadimitriou *et al.,* 2015). The adhesion property of probiotics seems to depend on the strain tested, the location of the colonic tissue and the age of the doner obtained, whereas theproperty of adhesion to the intestignal mucosa, depends on the origin obtained (human or animal) (Ouwehand *et al.,* 1999; 2001; 2002; Lee & Salaminien 2009). Probiotics often showa grater ability to adhere to mucus than to colonic tissues(Lee & Salaminien 2009).Moreover, as regards the adaptationability of probiotic strains, from genomic information, the presence of several molecules was revealed. These molecules are able to adhere to different components of the mucus andto exchange signals with the intestinal immune system furtherly (Salaminien *et al.*, 2005; Lee & Salaminien 2009).

Antimicrobial activity against potentially pathogenic bacteria

In some cases the antimicrobial activity depends on theprobiotic strain, the targeted pathogen strain and the chosen growth media to observe them(Collado *et al.,* 2005; Gueimonde *et al.,* 2006; Lee & Salaminien, 2009, Suskovic *et.al.,* 2010).In addition to that, the general mechanism of antimicrobial activity of bacteria has been attributed to the production of antimicrobial substances which are mainly acids, ethanol,hydrogen peroxide, proteins, lipidsand other low molecular mass compounds (Suskovic *et.al.,* 2010).Those specific mechanisms to be measured needs a case-by-case assays in order to select probiotics with the ability to inhibit or exclude certain pathogens (Lee & Salaminien, 2009). The amount and type of thoseexcreded products influences the subsequent antimicrobial activity in the fermented material or media. More information about the antimicrobial activity of industrially important lactic acid bacteria and probiotic bacteria can be found at the review of Suskovic *et.al.*(2010).

Ability to reduce pathogen adhesion to surfaces

In connection with the previous paragraph, the ability of probiotics to produce antimicrobials is one mechanism to inhibit, exclude or compete with adherent enteropathogens for the ecological niche.A potential mechanism for adhesion to the host implicates the binding of molecules exposed on the surface of microbial cells to the intestignal mucosa layer (Papadimitriou *et al.,* 2015).Using human intestinal mucus it has been revealed that the possibility of adhesion is strain dependend wereas the colonization of the strains is achived after antagonism with other potential pathogenic strains (Collado *et al.,* 2005; Gueimonde *et al.,* 2006; Lee & Salaminien, 2009). Mucus-binding proteins (Mubs), surface (S-) layer proteins and sortase-dependent surface proteins mainly,have an important role in the

adherence process of probiotic to the hosts' extracellular glycoprotein matrix. Most often, co-cultures of probiotic and enteropathogens are carried out to test the antimicrobial ability of probiotic strains (Hutt *et al.*, 2006; Huys *et al.,* 2006;Starhinic *et al.,* 2007; Lee & Salaminien, 2009), but a screening with bioinformatics tools can predict that adhensive potential former to *in vitro* and *ex vivo* tests (Papadimitriou *et al.,* 2015).

Bile salt hydrolase activity

Bile is a solution with bile acids, cholesterol, phospholipids, and the pigment biliverdin(Carey & Duane, 1994; Hoffman, 1994; Hill *et al.*, 2006, Kumar *et al.,* 2012). Bile ishaving an essential role in fat digestion also confers potent antimicrobial activity, primarily through the dissolution of bacterial membranes(Begley, Gahan & Hill, 2005; Hill *et al.,* 2006, Kumar *et al.,* 2012). Three genes encoding bile salt hydrolases and genes for bile transport to evade bile toxicity (Tamime *et.al.,* 2005). Deconjugation of bile is catalyzed by bile salt hydrolase (BSH) enzymes, which hydrolyze the amide bond and liberate the glycine/taurine moiety from the steroid core (Hill *et al.,* 2006, Kumar *et al.,* 2012). BSH is present in all *Bifidobacteria* and *Lactobacilli* strains associated with the gastrointestinal environment. Some species of probiotics are able to produce deconjugated bile acids, which are derivatives of bile salts (BA) and are able to suppress the growth of fungi and other species of bacteria (Hill*et al*.,2006).

Deconjugation is the process of fracturing the C-24 N-acylamide of conjugated bile acids, which links BA to glycine or taurine and generates unconjugated BA(Nie *et al.,* 2015).Bile salt deconjugation may therefore confer a nutritional advantage on hydrolytic strains (Hill *et al*.,2006). Deconjugated bile acids show a stronger antimicrobial activity compared to the bile salts synthesized from the human body(Oelschlaeger, 2010). Probiotics contribute to BA deconjugation in the ileum, BA secretion in the feces, and BA synthesis in the liver by suppressing the enterohepatic FXR-fibroblast growth factor 15 negative feedback mechanism of BA synthesis(Ettinger *et al.,* 2014, Nie *et al.,* 2015); a bile acid-activated receptor farnesoid X receptor (FXR; NR1H4); a receptor that regulates the transcription levels of critical genes in BA synthesis, transportation, and metabolism (Makishima *et al.,* 1999;Degirolamo *et al.,* 2014;Nie *et al.,* 2015). That receptor(Zollner *et al.,* 2006; Nguyen & Bouscarel, 2008; Nie *et al.,* 2015) is also involved in lipid, glucose and energy metabolism (Chiang, 2009).Moreover, Degirolamo *et al.,*(2014) observed an increase of Firmicutes and Actinobacteria after oral administration of probiotics and suppression of the Bacteroidetes and Proteobacteria phyla, leading to the elevation of BA excretion in the feces and hepatic BA synthesis. It has been proposed that secondary bile acids resulting from the subsequent modification of unconjugated bile salts may cause DNA damage, promote colon cancer, or result in impaired colonic mucosal function that may lead to diarrhea or inflammation (Kandell & Bernstein, 1991;Marteau *et al.,* 1995; Nagengast, Grobben, & Van Munster, 1995; Pazzi *et al.*, 1997; Bernstein *et al.,* 2005;Hill *et al.,* 2006).Bile salt hydrolyzing activity can be evaluated by *in vitro* tests and analytical chemistry techniques (Papadimitriou *et al.,* 2015).

Resistance to spermicides (applicable to probiotics for vaginal use)

From data obtained by microbiome studies, vaginal flora appears dominated by one or two species of *Lactobacillus*(Reid, 2005, Lamont *et al.,* 2011). Significant numbers of healthy women lack appreciable numbers of *lactobacilli*(Lamont *et al.,* 2011). If probiotic microorganisms are to be used in the urogenital tracts of sexually active women, their ability to survive in the presence of spermicides is crutial (FAO/WHO, 2001). Non-ionic spermicidal detergents, such as nonoxynol-9, can kill hydrogen peroxide producing *Lactobacillussp.* of the normal vaginal flora (Reid & Bruce, 2001),increase the pH and thusleading to overgrowth of gram-negative pathogenicmicroorganisms causing subsequent urogenital track infections(McGroarty *et.al.,*1992;Pascual *et al.* 2006)

Other Probiotic Properties;

In addition to the previously reviewed properties, other characteristics could be tested to consider a strain as potential probiotic. From these screenings it has been reported that some strains are able to modulate the immune system (Papadimitriou *et al.,* 2015), to produce antigenotoxic compounds(Caldini *et al.,*2005,Tiptiri-Kourpeti *et al.,* 2016), and to decrease cholesterol levels(Kumar *et al.,* 2012).

3rd Phase studies

The 3rd phase includes in vivo experiments in laboratory animals (mainly in mice and rats). In addition with *in vitro* and *ex vivo* assays, preclinical testing with probiotics can be performed in *in vivo* models by using laboratory animals or non-animal methods whenever possible(Lee & Salaminien, 2009).A sound knowledge of anatomy, physiology, and genetics is an advantage when selecting an animal model, since phylogenetic closeness is not always a guarantee for valid extrapolation (Lee & Salaminien, 2009). An ultimate selection of the animal model should be primarily based on how well the model explains the specific aims, rather than how well it represents the target (Lee & Salaminien, 2009, Papadimitriou *et al.,* 2015). In particular laboratory animal models can be divided into five different disease categories (Hau & van Hoosier, 2004 in Lee & Salaminien, 2009) depending on which disease condition meant to be investigated. Body size and metabolic rate should be also taken into account when selecting an animal model, as there are usually large differences between the model and the target species (e.g., mouse vs human) (Lee & Salaminien, 2009).

The policy of the implementation of the 3 Rs for replacement (use of alternative methods), reduction (use of minimum number of animals), and refinement (use of improved experimental procedures, high standards of animal welfare, etc.) (Shanks *et al.,* 2009; Salaminien *et al*., 2009, Papadimitriou *et al.,* 2015)are indispensable prerequisites in order to enhance the life-time experience of the animals (2010/63/EU).

Mice and rats are the most popular laboratory animal models used in research about probiotics, but several other species have been used as well. New animal models are continuously developed for use in the investigation of mechanisms of action, measurement of pharmacokinetics, diagnostic and therapeutic procedures, nutrition and metabolic diseases, and of the safety and efficacy of test substances, such as novel probiotics (Lee & Salaminien, 2009).

4th Phase studies

The 4th phase includehuman trials, is further divided into four stages of surveillance trials(FAO/WHO, 2002). Several of the *in vitro* tests can be correlated with *in vivo* studies using animal models, but probiotics for human consumption must be further validated with proper human studies covering both safety (phase 1 trials) and efficacy (phase 2 trials) aspects. Moreover the validation process of a probiotic intended to be used towards a targeted disease or condition, furthure validation is needed with effectiveness (phase 3 trials) studies. The last phase includes trials for the sureilance(phase 4 trials) prossess for the probiotic state to be given. Each phase is further analysed:

Safety trials

The 1st Phase for the clinical evaluation of probiotic properties includes an assessment for safety taking into account the "functional" or probiotic aspects. The requirements fora candidate strain to be characterized as safe andwithout contaminants in its delivery form, asthe FAO and WHO recommended, are:

1. Determination of antibiotic resistance patterns
2. Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation)
3. Assessment of side-effects during human studiesAsssessment of toxic by-products production (in cases when the strain under evaluation is belonging to a species that is a known mammalian toxin producer)
4. If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.
5. Epidemiological surveillance of adverse incidents in consumers (post-market)

There is a possibllity a plasmid-associated antibiotic resistance to spread to other species and genera(Lee & Salaminien, 2009, Ingvar *et al.* 2013). However, in most cases the resistance is not transferable and the species are also sensitive to antibiotics in clinical use. A decision strategy has been proposed (Courvalin *et al.,*2006 inLee & Salaminien, 2009) using molecular techniques to assess the risk of antibiotic resistance in bacterial strains. The steps are:

- identify the resistance gene;

- attempt to transfer resistance to normal gastrointestinal flora;
- characterize the biochemical mechanism of resistance;
- elucidate the genetic basis for resistance.

If after following this protocol the results showed that the resistance gene was not associated with a mobile genetic element, then the risk of transfer of resistance would be assessed as low (Courvalin *et al.,* 2006 in Lee & Salaminien, 2009). In addition, there are several genera with a long history of safe consumption in traditionally fermented products(WGO, 2011, EFSA, 2012)like *Enterococci sp.*. In addition, the species that are currently safe for use, are characterized with "General Recognised As Safe" (GRAS) status by the American Food and Drug Association (Hoffman *et al.,* 2012)or with a "Qualified Presumption of Safety" (QPS) consideration status by the European Food Safety Authority (EFSA)(Binns, 2013).Furthermore, EFSA has proposed a scheme based on the concept of QPS, defined as "an assumption based on reasonable evidence"(EFSA, 2012, SANCO, 2003). This scheme aims to have consistent generic safety assessment of microorganisms through the food chain without compromising safety standards.Broadly the characteristics to be evaluated for QPS approval are:

a. identification at the claimed taxonomic level;
b. relationship of taxonomic identity to existing or historic nomenclature;
c. degree of familiarity with organism, based on weight of evidence;
d. potential pathogenicity for humans and animals;
e. the end use of the microorganism.

The latter would influence any qualifications imposed, depending on whether the organism is to be directly consumed; is a component of a food product not intended to enter the food chain, but which may adventitiously; or is used as a production strain in a product intended to be free of other live organisms (Lee & Salaminien, 2009).

Efficacy trials

Phase 2 studiesmeasure the efficacy of the probiotic strain that should be designed as double-blind, randomized and compared with a placebo (DBPC)in order to determine and measure anypossible adverse effects (FAO/WHO, 2002).The principal outcome of efficacy studies on probiotics should provide benefits in human trials, such as improvement in condition, symptoms, signs, well-being or quality of life; reduced risk of disease or longer time to next occurrence; or faster recovery from an illness (Ganguly *et al.,* 2011). Each should have a proven correlation with the probiotic tested. A general recommendation is that the placebo must be comprized of the carrier devoid of the test probiotic (FAO/WHO, 2002). The selection of proper biomarkers of health and disease (Sanders, 2016)before the clinical endpoint is most

crucial. Reevaluation in another clinicalsample size needs to be calculated for specific endpoints. Statistically significant differences must apply to biologically relevant outcomes(Lovell, 2013). It is recommended that human trials be repeated by more than one center for confirmation of results (FAO/WHO, 2002).

Effectiveness trials

Phase 3 studies regard the evaluation of the medicinal effectiveness of the strain in which a comparison with a standard therapy takes place.When a claim is made for a probiotic altering a disease state, that claim should be based on sound scientific evidence in human subjects. Probiotics delivered in food form do not need substaination of efficacy(FAO/WHO, 2002).

Surveillance

The last phase of the evaluation process through the probioticsurveillance, regards cases where they are delivered in food form.It is recommended that those productsmust be properly labeled in compliance with the FAO/WHO guidelines(FAO/WHO, 2002). Like FAO/WHO guideline, ICMR/DBT guideline of India also deals with safety, health claim and labeling issues of probiotic products (Panwar *et al.,* 2016). Labels should include information about the identity of the organism(s); its GMO status; viability count and shelf life; dosing and duration; conditions for which its use is or is not appropriate; proven benefits; side effects, particularly symptoms that require clinical assessment; and the manufacturer's contact details(Lee & Salaminien 2009).

Currently, in most countries, only general health claims are allowed on foods containing probiotics. FAO/WHO recommends that specific health claims relating with the use of probiotics on foods are allowed where sufficient scientific evidence are available, as *per* the guidelines set forth inFAO/WHO report of 2001 and 2002. Such specific health claims should be permitted on the label and promotional material. For example, a specific claim that states that a probiotic 'reduces the incidence and severity of rotavirus diarrhea in infants' would be more informative to the consumer than a general claim that states 'improves gut health'(FAO/WHO, 2002). This must comply withCodex General Guidelines on Claims(Codex Alimentarius, 1979),General standard for the labelling and claims for prepackage food for special dieatary uses (Codex Alimentarius, 1985) and with the Guidelines for use of nutrition and health claims (Codex Alimentarius, 1997), as those revised and amended in the years followed, to avoid misleading information. FAO/WHO(2002)recommends that the following information have to be described on the label:

- Genus, species and strain designation. Strain designation should not mislead consumers about the functionality of the strain

- Minimum viable numbers of each probiotic strain at the end of the shelf-life
- The suggested serving size must deliver the effective dosage of probiotics related to the health claim
- Health claim(s)
- Proper storage conditions
- Corporate contact details for consumer information

Lastly, it is recommended that a verification process has to take place afterwards, from an independent third party scientific experts in the field, in order to establish that the health claims are truthful and not misleading.

2.1.4 Mode(s) of Action

Currently, the research on probiotic microorganisms, regarding their beneficial effects on host's health has emerged, yet little is known about every molecular mechanism or mechanism of the benefitsreported(Rijkers *et al.,* 2010, Gil *et al*., 2012, Hemarajata & Versalovic 2013). The mechanisms vary from one probiotic strain to another even for the same effect. The mechanism(s) may be a combination of events, including the production of an enzyme and possibly other metabolites which can act directly on a targeted pathogen microorganism or indirectly causing the hosts eucariotic cells to express a defending mechanism themselve, thus making this a very difficult and complex area to unravel (Vandenbergh, 1993; FAO/WHO, 2001, Hibbing *et al.,* 2010). On the basis of the FAO/WHO report, the possible mechanisms of action regarding the control of intestinal pathogens mainly include:

1. Antimicrobial substance production
2. Competitive exclusion of pathogen binding on the hosts epithelial barrier
3. Competition for nutrients
4. Modulation of the hosts immune system

Moreover, the International Life Sciences Institute (ILSI), which is affiliated with both WHO and FAO, published a consice monograph on the topic ‘probiotics, prebiotics and the gut microbiota’ in 2013 (Binns, 2013). Regarding the mode of action of probiotics two more possible mechanisms of action were reported, including:

5. Production of growth substrates for other bacteria

6. Improvement of gut barrier function

The mechanisms are illustrated below (**Fig. 2**)(O'Toole & Cooney, 2008)and in a different point of view by Rjikers *etal*. in 2010(Rijkers *et al.* for ILSI, 2010b), who depicked the potential mechanism of probiotic action in three layers and/or levels of action(**Fig. 3**.).

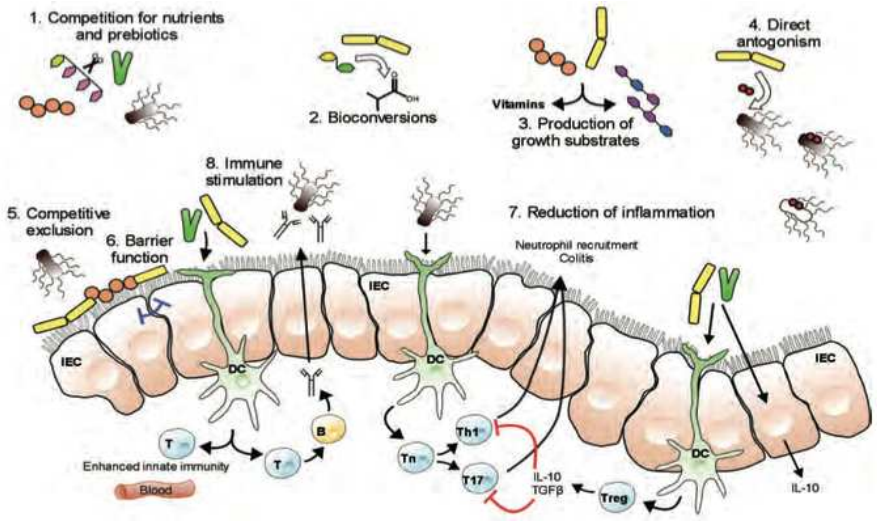

Figure 2: Potential or known mechanisms of probiotics action;
These mechanisms include (1) competition for dietary ingredients as growth substrates, (2) bioconversion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, for other bacteria, (4) direct antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colonisation and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC, epithelial cells; DC, dendritic cells; T, T cells.

Source: O'Toole and Cooney 2008 in ILSI EU Concise Monograph series, 2013

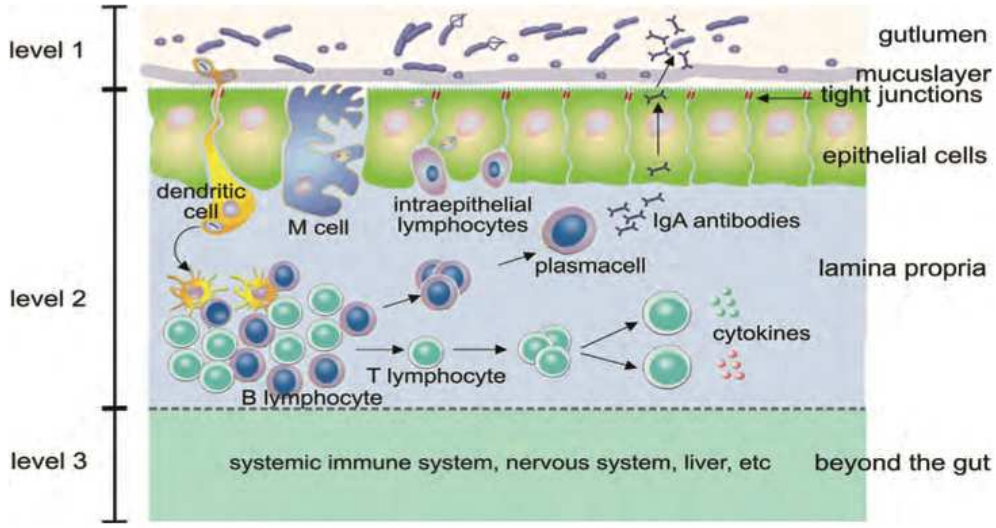

Figure 3: Levels of probiotic action
Probiotic bacteria can interfere with the growth or survival of pathogenic micro-organisms in the gut lumen (level 1), may improve the mucosal barrier function and mucosal immune system (level 2) and, beyond the gut, mighthave effect on the systemic immune system, as well as other cell and organ systems such as liver and brain (level 3). Source: Rijkers, 2010, ILSI EU Concise Monograph series, 2013

Production of antimicrobial substances

Today, a wide variety of strains including LAB, PAB, and bifidobacteria are industrially used as starter cultures, co-cultures, or bio-protective cultures to improve preservation, flavors, body and texture of dairy, vegetables, meats, and cereal products(Drosinos *et al.,* 2007, Nychas *et al*., 2010, Tassou *et al.,* 2014, Panagou *et al.,* 2014, 2016). The specific preservation method is based on the fermentation prossess in which the pH value is lowered, the amount of available carbohydrates is reduced, and many antimicrobial compounds are produced by fermenting bacteria.The production of antimicrobial substances against pathogenic microorganisms may be the most studied mechanism of action and can be characterized as a widespread or as a frequent mode of action among the studied probiotics (Sanders *et al.,* 2014).

Studies performed on probiotic bacteria showed that some of them can produce antibacterial compounds, which can lead to inhibition of pathogen adhesion and replication, mainly by affecting the target cell membrane(Gil *et al.,* 2012).(Therefore most of them can be characterized as a level 1 mode of action according to Rijkers(Rijkers, 2010; ILSI EU Concise Monograph series, 2013)).These components are almost always low-molecular-weight (LMW) compounds (< 1,000 Da), such asshort chain fatty acids (SCFA),organic acids, antimicrobial peptides (AMPs), bacteriocins (LMWB)and deconjugated bile acids.

Short chain fatty acids (SCFAs), which include acetate, propionate, and butyrate, are produced by bacteria in the gut during fermentation of insoluble fibre from dietary plant matter(Salazar *et al.*, 2016).

Organic acids (C1–C7)are widely distributed in nature as normal constituents of plants, animal tissues and can also be formed through microbial fermentation of carbohydrates mainly in the large intestine. However, there appear to have effects beyond antimicrobial activity. These include reduction in digesta pH, increased pancreatic secretion, and trophic effects on the gastrointestinal mucosa (Brul *et al.,*1999,Dibner &Buttin, 2002).

Antimicrobial peptides (AMPs) are small molecular weight proteins with broad spectrum antimicrobial activity against bacteria, viruses, and fungi. Cathelicidins and defensins are major groups of epidermal AMPs(Guani-Guerra *et al.,* 2010).Decreased levels of these peptides have been noted for patients with atopic dermatitis and in Kostmann's syndrome, altering the host immune response and function in angiogenesis, wound healing, and chemotaxis (Izadpanah & Gallo, 2005).

Bacteriocins are a heterogeneous group of particles with different morphological and biochemical entities. They range from a simple protein to a high molecular weight complexand are species specific. For the the classification, biochemical nature, morphology and mode of action of bacteriocins as well as their genetic determinants and the microbiological relevance of these bactericidal agents the papers of Daw & Falkiner 1996 and Suskovic *et al.* of 2010are suggested.

The enzymatic action of the bacterial flora converts the bile acids into secondary BAs, by removing the hydroxyl group from the 7th carbon atom on the molecule. The potential exists for altering the bile acid pool by targeting key enzymes in the 7α/β-dehydroxylation pathway through the development of pharmaceuticals or sequestering bile acids biologically in probiotic bacteria, which may result in their effective removal from the host after excretion. For further reading the paper of Hylemon *et al.* in 2006 is suggested.

Competitive exclusion of pathogen binding on the epithelial barrier

Another widespread mode of action of probiotics is their competitive mechanisms for binding sites and nutrients (Sanders *et al.,* 2014).

Mucosal surfaces employ a number of protective strategies to defend against noxious substances and pathogens found within the intestinal lumen. The intestinal epithelial cells lining the intestinal tract are a physical barrier and have evolved inducible innate protective strategies that offer rapid responses to pathogenic challenge (Ohland & Macnaughton, 2010). The anti-adhesive effect might be the result of several mechanisms. For example, they may act directly by physical blocking of the same receptors or indirectly by blocking the binding of pathogens, with the release of antimicrobial proteins (AMPs), such as α- and β-defensins, cathelicidins (Kelsall *et al.*, 2008; Rashidan *et al.* 2014), C-type lectins and ribonucleases implicated in the resistance of epithelial cell surfaces against a wide range of pathogenic bacteria fungi and viruses (O'Neil *et al.,* 1999; Takahashi *et al.,* 2001; Müller *et al.,* 2005; Furrie *et al.,* 2006;Rashidan *et al.,* 2014). An increase in mucin production is an innate protective strategy of the human body. Some strains can cause that reaction aswell as distict methods, such as; degradation of carbohydrate receptors by secreted proteins, biofilm formation,

production of receptor analogues and the induction of biosurfactants (Oelschlaeger, 2010) and alteration of tight junction signaling (Anderson *et al.,* 2010). Therefore that mechanism can be characterized as a level 1 mode of action according to Rijkers (Rijkers, 2010, ILSI EU Concise Monograph series, 2013).

However, the ability to inhibit the adhesion of pathogens to immobilized human mucus appears to depend on both the probiotic strain and the pathogen. Probiotic bacteria, such as *Lactobacillus, Bifidobacterium* and *Bacillus cereus* strains can produce auto-inducers that can control virulence gene expression in numerous microorganisms. For example, Griffiths *et al.* (2007)found out that *Lactobacillus acidophilus* La-5 secretes a compound that reduces the production of auto-inducer by *E. coli* O157 and through it, leads to significant reduction in the transcription of genes involved in colonization. Recently, many studies have reported similar results for *Bacillus cereus*(Medina-Martínez *et al.,* 2007; Rashidan *et al.,* 2014).

Competition for nutrient consumption

Probiotics may compete for nutrients and absorption sites with pathogenic bacteria in the gut. In addition, competition for energy and nutrients between probiotic and other bacteria may result in a suppression of pathogenic species. The gut is such a rich source of nutrients that it may seem unlikely that microorganisms could not find sufficient food for growth there(Hemarajata & Versalovic 2013). Probiotics possess a high fermentative activity and stimulate digestion(Cho *et al.,*2011)

Lactobacilli are able to produce lactic acid and proteolytic enzymes which can enhance nutrient digestion in the gastrointestinal tract. Different studies demonstrated that probiotics maximized crude protein and energy digestibility compared with those in non-probiotic treatments (Yirga, 2015).

However, it should be noted that the environment only has to be deficient in one essentiall nutrient in order to inhibit microbial growth. In addition, the ability to rapidly utilize an energy source may reduce the log phase of bacterial growth and make it impossible for the organism to resist to the flushing effect exerted by peristalsis (Yirga, 2015).Microbial growth requires suitable environmental conditions, a source of energy, andnourishment. These requirements can be divided into physical and chemical.

Bacteria communicate with each other and their surrounding environment through chemical signaling molecules that are called auto-inducers. This phenomenon known as quorum sensing (QS) can measure the population density, nutrient concentration and other ecological characteristicsin food systems as they may have a role in spoilage, growth and toxin production from pathogens present in food, bacteriocin production, and virulence responses (Medina-Martínez *et al.,* 2007). After those signals come into a critical level, QS can triger gene expressionamong its inducers into forminga more stable ‘community’ (biofilm) and secure microenviroment from bacterial antagonism (Gram *et al.,* 2002; Medina-Martínez *et al.,* 2007; Hibbing *et al*., 2010; OECD, 2011;Rashidan *et al*., 2014; Papadimitriou *et al*., 2015).

Immune system modulation

According to FAO/WHO (2001), the main likely mechanism of action of probiotics may be specific enzymes or metabolites that can act directly or indirectly in effecting the hosts immune system. The probiotic immunomodulatory effect even between the same genus is strain-dependent, and that the probiotic effects on immune responses appear to be immune regulating/modulating rather than immune activating/ stimulating (Hemarajata & Versalovic 2013; Panwar *et al.,* 2016)

In humans, the immune system functions take place in the blood, lymphatic system, thymus, spleen, skin, and mucosa tissues. The bacterial activity in the gut is considered as a major component for health,sincea major 80% of the immune system functions derive from gut flora (Sanders *et al.,* 2007).

Disfunction of components of the innate immune system and/or a disruption in the intestinal barrier function may result to enteric infections, celiac disease or to autoimmune diseases (Müller *et al.,* 2005; Parvez *et al.,* 2006; Sanders *et al.,* 2007; W. Allan Walker 2008; WGO 2008; WGO 2011; Vyas & Ranganathan 2012; Rashidan *et al.,* 2014; Szilagyi 2015).Probiotic bacteria appear to derive immune modulating effectson numerous cell types involved in the innate and adaptive immune responses such as epithelial cells, dendritic cells, monocytes/macrophages, B cells, T cells, regulatory T cells and natural killer (NK) cells and thereby exert their immunomodulatory effect. A review on the immune responces of lactic acid bacteria on the mentioned cells has conducted from (Zhong, Zhang & Covasa, 2014)and it is suggested for further reading.

2.1.5 Positive effects of probiotics on humans' health

Today the primary clinical interest in the application of probiotics has been the prevention and treatment of gastrointestinal infections and diseases(Parvez *et al*. 2006), since the early establishment of a healthy gut microflora in newborn infants is connected with long-term-health benefits(ILSI, 2010; Kim *et al*. 2006; Aceti *et al*. 2015).

Based on research reports and clinical intervention studies, it has been proved and widely accepted that all probiotic health effects are strain-, dose-, disease-, and possibly host-dependent(Hill *et al.,* 2014). Those three levels of action can be extrapolated from the first level in a widespred mode of actions performed from almost all probiotic strains, and may undergo a specific one, which is rare-and strain dependent, or a so called third level of action (**Fig.4**).

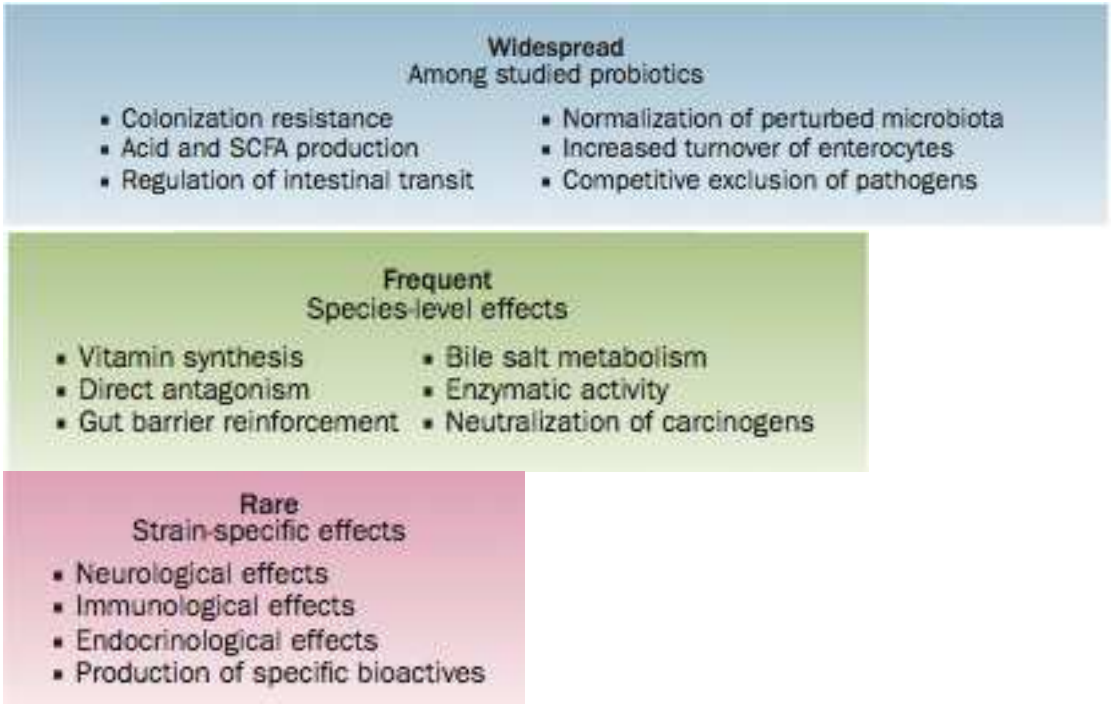

Figure 4: The three probiotic categories that ISAPP identified

Some mechanisms might be widespread among commonly studied probiotic genera; others might be frequently observed among most strains of a probiotic species; others may be rare and present in only a few strains of a given species. Evidence is accumulating on a cross-section of probiotic strains that suggest some generalizations can be made beyond strain-specific effects (Hill *et al.,* 2014 for ISAPP)

As laboratories around the world continue to carry out probiotics research, the experts say not every study will fit neatly into one of the three probiotic categories that ISAPP identified: (1) Well-known probiotic species that provide general health benefits, (2) Probiotic

strains that treat specific pathologies, and (3) Human commensal-derived probiotics used therapeutically like drugs.In summary, their applications seem to have a positive effect on:

General Health; after Consumption Of Traditional Fermended Foods

Lactic acid bacteria, in which probiotic bacteria are included, have been consumed for studied for their ability and their potential impact on the metabolism of dietary components in the small intestine lumen including lactose digestion, metabolism of lipids, such as cholesterol metabolism(Jain & Yadav *et al.,* 2012, Vyas & Ranganathan 2012, Ettinger *et al.,* 2014, Tsai *et al.,* 2014, Anandharaj *et al.,* 2014, Nie *et al.,* 2015, Papadimitriou *et al.,* 2015). In the large intestine, they contribute to the metabolism of otherwise undigestible dietary carbohydrates (e.g., prebiotics) and have a favorable effect on colonic protein and ammonia metabolism(ILSI *et al.,* 2010b). Probiotics also influence metabolism in the host tissues, in particular at the gastrointestinal mucosa and the liver(ILSI, 2010b,Zhong *et al.* 2014).

Production of vitamins

Vitamins are involved in many essential functions of the body like cell metabolism, synthesis of nucleic acids and antioxidant activities (Patel *et al.,* 2013). Most of the vitamins cannot be synthesized by humans and animals; several species of bacteria, may serve to produce folic acid, vitamin B12 or cobalamin, vitamin K2 or menaguino, riboflavin, thiamine, biotin and other essential vitamins (Tamime, 2005, Kim *et al.,* 2006, Rossi *et al.,* 2011, Rijkers *et al.,* 2011, Serraj & Andres, 2012, Patel *et al.,* 2013, Ettinger *et al.,* 2014, Aragón, 2014, Papadimitriou *et al.,* 2015).

Hypercholesterolemia

Several studies have reported that the consumption of fermented dairy products can decrease serum cholesterol(Anandharaj *et al.,* 2014). There are few possible proposed mechanisms that resut in cholesterol reduction by probioticsincluding assimilation of cholesterol, however, some of these mechanisms are strain dependent(Jain & Yadav *et al.,* 2012, C.R Soccol *et al.,* 2013, Anandharaj *et al.,* 2014, Shimizu *et al.,* 2015, Papadimitriou *et al.,* 2015), and conditions generated in the laboratory environment, would not be practical in the *in vivo* systems (Jain & Yadav *et al.,* 2012). Even though the hypocholesterolemic mechanism of probiotics has not yet been fully understood, it is an established fact that cholesterol and bile salt metabolism are closely linked (Jain & Yadav *et al.,* 2012, Anandharaj *et al.,* 2014).

Folic acid/ Vitamin B9 Deficiency

Dietary folate cannot be synthesized by the mammalian cells and must be obtained from exogenous sources like foods or the intestinal microbiota. Folate deficiencies are associated with a variety of disorders such as osteoporosis, Alzheimer' s disease, coronary heart disease and increased risk of breast and colorectal cancer as indicated from epidemiological studies (Rossi *et al.,* 2011, Patel *et al.,* 2013).Wouters *et al.,* (2002) suggested that in yogurts the amount of folate may be increased depending on the starter culture (in Patel *et al.,* 2013).

Pernicious Anemia/ Biermer's diseaseor Cobalamin/ Vitamin B12Deficiency

Vitamin B12 cannot be synthesized by mammals and must be obtained from exogenous sources like foods or the intestinal microbiota (Patel *et al.,* 2013).Is required as a co-factor for the metabolism of fatty acids, amino acids, nucleic acids and carbohydrates (Tamime, 2005, Patel *et al.,* 2013).Deficiency in slightly lowerthan normal levels, symptoms offatigue, depression, and poor memory may appear (Patel *et al.,* 2013). In addition long-time vitamin B12 deficiency can potentially cause severe and irreversible damage, especially to the brain and nervous and haematopoietic systems(Chambers *et al.,* 2000;Patel *et al.,* 2013).Such as Pernicious Anemia (PA), a type of blood disorder. Pernicious anemia (also known as Biermer's disease) is an autoimmune atrophic gastritis, predominantly of the fundus. Biologically, it is characterized by the presence of anti-intrinsic factor antibodies in which patients cannot produce enough intrinsic factorin their stomach. Treatment is based on the administration of parenteral vitamin B12, although other routes of administration (eg, oral) seems to be effective if given in high doses.Recently it was revealed that the pathogen *Helicobacter pylori* seems to contributesin the pathogenesis of autoimmune gastritis and PA (Andrès 2012, Serraj & Andres 2012, Patel *et al.,* 2013)

Eczema

Atopic dermatitis, atopic eczema or eczema in children, is a chronic dermatological disorder.Prevailence of allergic diseases in infants, whose parents and siblings do not have allergy, is approximately 10% and reaches 20–30% in those with an allergic first-degree relative (Muraro *et al.,* 2014, Aceti *et al.,* 2015).In the FAO/WHO guidelines of 2001, forthe modulation of the immune response and for the prevention of allergic diseases, isreferred on infant subjects only and further it was achieved with ingestion of the strains *L. rhamnosus* GG and *B. lactis* BB-12 (Majamaa & Isolauri, 1996; 1997; Kalliomaki *et al.,* 2001; Isolauri *et al.,* 2000).The World Allergy Organization (WAO) convened a guideline panel to develop evidence-based recommendations about the use of probiotics in the prevention of allergy in 2015(Brożek *et al.,* 2015).The guideline panel suggests using probiotics ininfants who havea biological parent or sibling with existing or history of allergic rhinitis, asthma, eczema, or food allergy as well as during the last 3 months of pregnancyin women of high risk for allergy transmition and/or during the breastfeeding period may prevent atopy(Brożek *et al.,* 2015). In addition The World

Gastroenterology Organization (WGO) in its Global Guidelines of 2011 establishes that “the strongest evidence is refered to the prevention of atopic dermatitis when certain probiotics are administered to pregnant mothers and newborns up to 6 months of age ” although the supported evidence were considered asvery low in quality.(WGO, 2011)

Lactose sensitivity/Maldigestion/ Malabsorption

In adults the ability to digest lactose is a dominant trait known as lactase persistence (LP). Those who cannot digest lactose (recessive trait) are described as lactase nonpersistent (LNP). Diseases affecting the brush border (eg, celiac disease, Giardia, bacterial overgrowth, viral gastroenteritis, radiation and others) can lead to secondary lactose maldigestion in adulthood (Szilagyi, 2015). Flatz and Rotthauwe suggested that due to lack of sunshine and skin synthesis of vitamin D, the inability to digest lactose allowed greater amounts of calcium assimilation from dairy foods, especially raw milk, causing symptoms such as gas, ache and additional symptoms beyond the above outlined gastrointestinal symptoms such as headaches and depression (Flatz & Rotthauwe, 1973;Szilagyi, 2015). Lactose sensitivity is increased but also becomes independent of race and ethnicity in patients with inflammatory bowel disease (IBD) (Mishkin, 1997;Eadala *et al.,* 2011Szilagyi, 2015). Moreover, some of these persons meet criteria for Irritatable Bowl Syndrome (IBS)and also may have other carbohydrate sensitivities.

In general, diseases that would be affected by larger intakes of dairy foods (and in which the putative pathogenic factor is not lactose) could also impact adapted LNP persons who are now able to consume larger quantities of dairy products (for example, diseases such as prostate and testicular cancer). However, diseases in which a favourably modified intestinal flora may act against the disease, dairy food and/or milk-consuming LNP persons may be somewhat protected (eg, diseases such as colrectal cancer and bladder cancer) (Szilagyi, 2015).

Lactose maldigestion depends on the race and the geographical region of living, the sunshine exposure (a.k.a vitamin D availability). The porpotions of lactace intake are crucial for a LNP occurance (Szilagyi, 2015). This is defined by an increase in blood glucose concentration of <1.12 mmol/Lor breath hydrogen of >20 ppm after ingestion of 1 g/kg body weight or 50 g lactose (Liong, 2011, Harris &Bayless *et al,* 1989). Both natural (de Vrese *et al.,* 2001, Guarner *et al.,* 2005) and denatured (pasteurized, heated) yogurts improved lactose digestion in lactose maldigesters reducing that effect on the microbial b-galactosidase (heat-sensitive) and on the exogenousgalactosidase (lactase enzyme) preparations (de Vrese *et al.,* 2001) The delay of lactose in gastrointestinal transit prolongs the action of residual b-galactosidase and decreases osmotic loadof the lactose (bloating) in an *in vivo* study on lactase deficient (de Vrese *et al.,* 2001). To effectively release galactosidase,bacteria need an intact cell wall as mechanical protection of the enzyme during gastric passage and against the action of bile. It was demonstrated that gastric acid degrades bacterial lactase activity in 20-60 min.

Lactose Intolerance

Not all studies confirm the efficacy of oral probiotics in adults with lactose intolerance (Szilagyi, 2015). Viable yogurt starter cultures have the potential to improve lactose digestion and eliminate symptoms. The Lactose Intolerance Global Network (LIGN) and the Lactose Intolerance Spanish Association (ADILAC) assessed a practical guide called "The milky life: The Practical Guide on Lactose Intolerance" with 100 questions and answers covering about all currently available information concerning this food intolerance. This guide is suggesting the consumption of the genera *Lactobacillus, Bifidobacterium* and *Acidophilus* in alleviating clinical symptoms brought about by undigested lactose or by other reasons. Evidence suggest that the probiotic effects on lowering lactose intolerance complications are not only strain-specific, but concentrations and preparations are also crucial. Moreover, the composition of colonic microflora, the gastrointestinal conditions (e.g, pH) (de Vrese *et al.,* 2001), the race and the age are also important influences regarding its occurrence (Wilt *et al.,* 2010).For further reading the National Osteoporosis Foundation (NOF) link is suggested at www.nof.org.

Dental caries /tooth decay

The oral cavity shelters a numerous and variable microbial flora (Badet & Thebaud 2008). One major aftereffect of this complex ecosystem, is the dental plaque which develops naturally on oral tissues (Badet & Thebaud 2008). The bacteria in plaque can cause tooth decay and gum disease if they are not removed regularly through brushing and flossing.

Tooth decay, is a chronic disease affecting both the crowns and roots of teeth. Dental caries forms through a complex interaction over time between cariogenic microorganisms; acid-producing bacteria(mainly mutans *streptococci spp*. and *lactobacilli sp*.) or yeast overgrowth (mainly *Candida spp*.) in the elderly, fermentable carbohydrate, and many host factors including inadequate salivary flow, insufficient fluoride exposure, poor oral hygiene, inappropriate methods of feeding during infancy, and poverty (Selwitz *et al.,* 2007, Badet & Thebaud 2008,Di Pierro *et al.,* 2015).

Mutans *streptococci* have been considered for a long time the major pathogens involved in caries (Selwitz *et al.,* 2007, Di Pierro *et al.,* 2015). Temporary colonization of probiotic bacteria in the oral cavity may competitively inhibit the caries pathogens due to the capability to form biofilm and produce acids, but that ability to colonize in the mouth is still under evaluation. Supplementation with *Streptococcus salivarius* spp., may potentially help in limiting the progression of dental caries by reducing plaque accumulation and plaque acidification, in both children and adults respectively (Di Pierro *et al.,* 2015). The European Academy of Paediatric Dentistry (EAPD) suggests the *Bacillus coagulans* for further research in prevention of caries in children, as it seems that it significately reduced ($p<0.001$) mutans *streptococci* colony counts per ml of saliva (Jindal *et al.,* 2011).

Human commensal-derived probiotics used therapeutically as drugs

Prevention and Treatment of Diarrheal diseases

Each year about 1.7 billion casesand around 760.000 deaths of children under fivedue to diarrhea episodes occur worldwide.In up to 15% of cases, diarrhea may be prolonged (1 week to 1 month, rarely up to 1 year) and associated with repeated bouts of abdominal cramping, malaise, nausea, fever, or muscle pain (WHO, 2013a). Diarrhea is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms and itis spread through contaminated food or drinking-water, or from person-to-person as a result of poor hygiene (Manary *et al.,* 2012). Diarrhea is defined as 4–6 loose, watery, or bloody bowel movements per day.

WHO in the 2013Guideline: "Updates on the management of severe acute malnutrition in infants and children" reports that probiotics have some effect in well-nourished children on diarrheal morbiditygiven ahigh-dose *Lactobacillus rhamnosus GG* (but the findings on malnourished children have been inconclusiveabout Acute Rotavirus Diarrhea, suggesting that more research is needed(WHO, 2013b).

More specifically there are few categories of diarrheal diseases.**Antibiotic-Associated Diarrhea;** patients under treatment with antibiotics may develop *Clostridium difficile* colitis diarrhea or due to the rapid emergence of antibiotic-resistant pathogenic strains(Tamime, 2005, de Vrese & Marteau, 2007, Hempel *et al.,* 2011, WGO 2011). Probiotic strains have shown to exert a protective effect at least against **Radiation-Induced Diarrhea;** radiation therapy disturbs the indigenous intestinal microbiota, which may lead to acute enteritis and colitis (Lee & Salaminien 2009, WGO 2011, Amara & Shibl 2013). **Traveler's Diarrhea;** a common health complaint among travelers that usually lasts for 2–6 days if untreated(Ohashi & Ushida 2009, Lee & Salaminien 2009, Amara & Shibl 2013), **Diarrhea in Tube-Fed Patients** caused by overgrowth of enteropathogens(Tamime 2005; de Vrese & Marteau 2007; Lee & Salaminien 2009)without an increase in clinically important adverse events. Other clinicaly important diaseases are analyzed below.

Irritable Bowel Syndrome

Irritable Bowel Syndrome IBS is a functional bowel disorder manifested by chronic, recurring, abdominal pain or discomfort,characterized by functional abdominal pain with diarrhea or constipation or mixed bowel habits or other insufficient stool consistency in the absence of structural and biochemical abnormalities(Haller *et al.,* 2010, WGO, 2011, Jung *et al.,* 2014, WGO, 2015). The cause of IBS occurance is yet unknown but altered gut motility, visceral hypersensitivity, psychological factors and disregulation of the brain–gut axis may be among the trigger mechanisms(Lee & Salaminien 2009, WGO, 2015).

Probiotic strains, applied either singly or in combination, appeared effective in relieving some of the IBS symptoms, such as constipation, flatulence, and borborygmi, but the

effects and the efficacy varied widely between studies and between strains of probiotics due the variable course of IBS symptoms, the heterogeneity of the condition, and the very high placebo response (up to 50%)(Haller *et al.,* 2010).Since several strains are conflicated, and there is about a 50% possibility of treatment, The International Foundation For Gastrointestignal Disorders (IFFGD) suggests *Bifidobacterium sp.* (since *Bifidobacterium infantis*mainly appear to have a beneficial effect in gas and bloating symptoms of IBS),patients to individually estimate, document and compare their IBS symptoms, at weekly scale, which probiotic benefits best their symptoms. On the other hand WGO in '*WGO handbook on gut microbes'* of 2014 evaluates that synbiotics (a combination of pro- and prebiotics) treatment was most effective in alleviating IBS symptoms(Guarner *et al.,* 2014).

Inflammatory Bowel Diseases (IBD)

Infammatory Bowel Diasease (IBD) became an important disease in the past 75 years and its rates are increasing in Westernized societies. Also, IBD has been linked both with sunshine, vitamin D and lactase distributions. In general, UC precedes CD by approximately 15 to 20 years (Szilagyi, 2015, and references therein).

IBDis characteraized as hyper-sensitive-reaction towards bacterial antigens leading to a chronic relapsing diasese. (Mikov *et al.,* 2014) There are two major types of IBD, Ulcerative colitis (UC) and Crohn's disease (CD). UC is manifested exclusively in colonic mucosa while CD shows symptoms such as granulomas and intestinal fibrosis throughout all areas of the gastrointestinal (GI) tract (Jin *et al.,* 2014). The underlying mechanisms and factors that sustain activation of the mucosal immune system resulting in an active inflammation, are still unknown.In summary, manipulation of intestinal microbiome represents a promising type of therapy for IBD, which may lead to long-lasting remission for patients overcoming corticoids (Mikov *et al.,* 2014, Jin *et al.,* 2014). It has been proved that specific probiotic strains, can prevent intestinal inflammation and may possibly treat active IBD, probiotics and synbiotics has positive effects in the treatment and maintenance of UC whereas in CD clear effectiveness has only been shown for synbiotics(Gil *et al.,* 2014). Clinical surgery is often suggested but in most cases may not eliminate the extra-intestinal symptoms. For examplepouchitis and cholangitis may further occure. In those cases,multispecies probiotics mixture of LAB and probiotic bifidobacteria may improve the associated IBD symptoms, mainly for UC (WGO 2015), in mild CD (WGO, 2014) and for the cases of assossiated pathologies (Gil*et al.,* 2015, WGO,2014,2015).

Helicobacter pylori Infection

Helicobacter pylori is a spiral shaped bacteria that can grow in the mucus layer of the stomach.Infection of gastrointestinal tract is linked to the development of gastric and duodenal ulcers, gastric cancer, atrophic gastritis, mucosa-associated lymphoma tissue lymphoma, and other gastric complications like gastroesophageal reflux disease (GERD) and

dyspepsiabut some cases are asymptomatic(Kusters *et al.,* 2006). Importantly from 2012, it is considered a Class 1 carcinogen in the list of the International Agency for Research on Cancer (IARC). The recommended treatment for the eradication of *H.pylori* is a combination of proton-pump inhibitor and antibiotics.These manifestations are related to the changes in the intestinal microbiota profile. Probiotic administration in *H.pylori*eradication is questionable(Homan & Orel 2015). Several studies reported that certain probiotic bacteria exhibit inhibitory effect against *H. pylori in vitro* and *in vivo*(González *et al.,*2014).In contrast, other studies report that probiotics can only improve the adverse side effects of diarrhea and nausea (Yu *et al*. 2016). Furthermore, Lv et.al. suggested that the administration of *Lactobacillus* sp. or multiple probiotic strains, prior or subsequent with a standar therapy for over 2 weeks,improved the eradication efficacy and reduced the adverse effects during therapy (Xie *et al*. 2015).

Gastrointestinal Symptoms from Human Immunodeficiency syndrome (HIV) / Acquired Immunodeficiency Syndrome (AIDS)

The FAO publisheda book named *Milk and Dairy Products in Human Nutrition* in 2013(FAO, 2013). Interestingly, it has been reported that yoghurt consumption can benefit vulnerable populations, including fighting with the human immunodeficiency virus (HIV) (Lee & Salaminien 2009) by improving nutritional intake, gastrointestinal symptoms, compliance and tolerance of the anti-retroviral therapy (Solis *et al.,* 2002, Reid, 2010, FAO, 2013) among a sample of people living with HIV in Mwanza, Tanzania (Irvine, Hummelen & Hekmat, 2011). Data from the 24-hour dietary recall, conducted during the study, suggested that consumers of probiotic yoghurt had higher total energy and protein intakes and were more likely to achieve the recommended daily intakes of vitamin A, riboflavin, folate and calcium. However, the authors remarked that the results of this study needed to be further substantiated because of limits imposed by the observational, retrospective study design (Irvine, Hummelen & Hekmat, 2011).

Dols *et al.* (2011), in a randomized double-blind study, on the impact of probiotic yoghurt on HIV-positive women, found that yoghurt has the potential to transfer health benefits to the gut and participants revealed better appetite and less stomach gas. Anukam *et al.*(2008) suggested that yoghurt supplemented with *Lactobacillus rhamnosus* and *Lactobacillus reuteri* resolved moderate diarrhoea, flatulence and nausea in adult female patients with HIV/AIDS in Nigeria.Consumption of probiotic yoghurt was also associated with enhancement of the immune status by increasing the CD4 count of cells in HIV positive subjects (Irvine Hummelen & Hekmat, Reid 2010, FAO, 2013).

Anti-tumor Effects

Cancer occurance is related with multiple genetic polymorphisms triggerd bydivide self and non-self chosen lifestyle(environment and dietary) factors.The environmental component

is implicated inabout 80% of all cancers including'*Western'* type life-stylehabits andexposure to certain microorganisms(Szilagyi 2015, WGO, 2011). Probiotic bacteria, along with dairy foods and dietary ingredients, help in detoxification and biotransformation of procarcinogens and carcinogens into less toxic metabolites and thus prevent tumor formationmainly by modulation of the host's immune responses(Raman *et al.,* 2013). There are several studies on the anti-neoplastic mechanistic effects of probiotics in *in vitro* and in animal models, without causing more adverse events (Raman *et al*. 2013; Tiptiri-Kourpeti *et al*. 2016). As for clinical trials that demonstrate the antitumor effects of probiotics in humans, there are some randomized clinical controlled study trials and epidemiological studies,for the reduction of reaccurance or risk of developing cancer (De Roos & Katan 2000, Aragón 2014)

Cardiovascular diseases

Cardiovascular diseases (CVDs) include diseases of the heart, vascular diseases of the brain and diseases of blood vessels (WHO, WHF & WSO, 2011).Tobacco smoking, physical inactivity, unhealthy diet habbits and extensive alcohol consumption are the main behavioural risk factors of CVDs to occur. These risk factors are shared by other major NCDs such as cancer, diabetes and chronic respiratory disease(WHO, WHF & WSO 2011). Long-term exposure to behavioural risk factors results in raised blood pressure (hypertension), raised blood sugar (diabetes), raised and abnormal blood lipids (dyslipidaemia) and obesity. Major cardiovascular risk factors such as hypertension and diabetes link CVD to renal disease(WHO, WHF & WSO 2011).

In terms of attributable deaths, the leading cardiovascular risk factor globally is raised blood pressure (to which 13% of global deaths is attributed), followed by tobacco use (9%), raised blood glucose (6%), physical inactivity (6%) and overweight and obesity (5%)(WHO, WHF & WSO 2011).

Unhealthy diets such as those high in fat, salt, and free sugar and low in complex carbohydrates lead to an increased risk of cardiovascular diseases as WHO delineated (WHO, WHF & WSO, 2011). Indeed, high levels oflow density lipoproteins in the bloodstream can form plaque in the artery walls and therby is a causative factor for a cardiovascular disease incidence such as development of atherosclerosis, coronary heart disease, and stroke(Anandharaj *et al.,* 2014).The World Health Organization (WHO) in collaboration with the World Heart Federation (WHF) and the World Stroke Organization (WSO)has predicted that by 2030, CVD will remain the leading cause of death and will affect approximately 23.6 million people globally(WHO, WHF & WSO, 2011). That's why prevention methods isa major concern(Anandharaj *et al.,* 2014)taking into account that the available long term therapeutic use of anti-hypertensive drugs, are implicated with several undesirable side effects(Kumar *et al.,* 2012; Anandharaj *et al.,* 2014).Combination of anti-hypertensive drug therapy with probiotic consumption, since they have the potential in reducing cholesterol (Kumar *et al.,* 2012; Anandharaj *et al.,* 2014) for example using strains inoculating the angiotensin converting enzyme (ACE)-inhibitory(Reid *et al.,* 2014) .Although, the World Heart Federation (WHF) and

the World Stroke Organization (WSO) are not listing probiotics as a protective and therapeutic effect of probiotics against myocardial infarction and heart failure.Some of the clinical applicated examples of strains are listed below (**Table 1**).

Table 1: Different type of probiotics, and their clinical effects considering humans

Disease name	**Strain**	**References**
Eczema	*Escherichia coli, Bifidobacterium bifidum, Bifidobacterium lactis, Lactococcus lactis,L. rhamnosus GG*	Sanders *et al.,* 2007, Vyas & Ranganathan 2012,Amara & Shibl 2013 and references therein
Immunity enhancement	*Bacillus circulans, Lactobacillus plantarum, Lactobacillus casei, AJ2 (combination of eight Gram-positivespp.)*	Kim *et al.,* 2006, Rossi *et al.,* 2011, Ferlazzo *et al.* 2011,Amara & Shibl 2013 and references therein, Bui *et al.* 2015, Tiptiri-Kourpeti *et al.,* 2016
antibiotic-associated diarrhea	*Enterococcus mundtii,Lactobacillus GG, Lactobacillus plantarum, Lactobacillus brevis,L. rhamnosus GG, Staphylococcus boulardii strain of S. cerevisiae*	Timmerman *et al.* 2004, Nader-Macías *et al*. 2008,WGO 2011, Amara & Shibl 2013 and references therein, Preidis && Versalovic 2014
Gastroenteritis	*B. lactis Bb-12, Lactobacillus casei GG, L. reuteri and L. casei Shirota in fermented milk Lactobacillus casei,Saccharomyces boulardii*	Ruiz *et al*. 2005,WGO 2011, Amara & Shibl 2013 and references therein,Preidis& Versalovic 2014
Vaginal candidiasis (thrush)	*Lactobacillus rhamnosus GR-1 , Lactobacillus reuteri RC-14* *L acidophilus*	Reid & Bruce 2001, 2003, Martinez *et al*. 2009
Urinary tract infection	*Lactobacillus rhamnosus, Lb. reuteri,and Lactobacillus fermentum RC-14*	,Reid & Bruce 2001, 2003, Griffiths *et al*. 2007, Amara & Shibl 2013 and references therein
Lactose intolerance	*Lactobacillus acidophulus,Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus*	de Vrese *et al*. 2001,Guarner *et al*. 2005,WGO 2011, Amara & Shibl 2013 and references therein
Symptoms of Irritable bowel syndrome	*Bifidobacterium infantis sp.,Bifidobacterium lactis DN-173010 Escherichia coli DSM17252 Bifidobacterium infantis 35624*	Amara & Shibl 2013 and references therein, Gil *et al*. 2014,WGO 2015
Traveler's diarrhea	*Lactobacillus GG,Lactobacillus plantarum, Saccharomyces boulardii*	de Vrese & Marteau 2007,Collignon *et al*. 2008,Amara & Shibl, 2013 and ref. therein

Disease name	Strain	References
Radiation-induced diarrhea	*VSL#3,*	WGO 2011
Maintanace of remission Crohn's disease	*Escherichia coli strain Nissle 1917,* *Saccharomyces boulardii,B. longum,*	Calafiore *et al*. 2012,Butel 2013, Ghouri *et al*. 2014
Ulcerative colitis	*Lactobacillus acidophilus Escherichia coli Nissle 1917, VSL#3 Bifidobacterium,*	Amara & Shibl, 2013 and ref. therein, Jin *et al*. 2014
Peptic ulcer disease	*Lactobacillus acidophulus, S. boulardii,L. reuteri and L. GG*	González *et al*., 2014,Homan & Orel 2015
Hypercholesterolemia and cardiovascular diseases	*Enterococcus faecium, Lactobacillus plantarum,Lactobacillus acidophilus, L. plantarum, Propionibacterium freudenreichii, PROBIOS-23 Complex*	Ooi & Liong 2010,Amara & Shibl, 2013 and ref. therein, Tsai *et al*. 2014, Shimizu *et al*. 2015

Considering that probiotics often act in host-depended manner, Canadian companies of interest conducted an open-database in 2010, informing probiotic strains that may have a positive impact on different health indications. Most importantly, the chart details the level of evidence that support the use of that product for various adult and pediatric health indications based on the available publised clinical trials. Interestingly, even though FDA does not recognize the probiotic statement and recently a simmilar open database conducted by american companies of interest was released. Both databases can be found at http://www.aeprobio.com. For further information, there are several foundations and non-profit collaborations of scientists, and other associations, with listed benchmark documents on their webpages such as the ILSI, ISAPP, IPA.

It is an obvious requirement that a probiotic should not cause infection. However, probiotics may theoretically be responsible for several side-effects mainly in non-healthy subjects(OECD 2011, EFSA 2012, EFSA 2013) (**Table 2**). This is a significant issue where the intestinal barrier is immature as in infants; where its integrity is impaired from radiotherapy, antibiotic treatment or disease; and in immunocompromized states (Lee & Salaminien, 2009).

Table 2: Potential pathogenicity of probiotic microorganisms mostly used today

Genus	Strain	Potential complications/infections	References
Lactobacillus	*L.rhamnosus,* *L.casei,*	Mostly non-pathogenic, possible infections like endocarditis and bacteremia in people who had use of catheter and in a prosthetic joint, in	OECD 2011, EFSA 2012, 2013

	L. paracasei, *L. plantarum* *L. delbrueckii* *L.iners* *L.gasseri* *L. GG*	immunocompromised patients with severe active ulcerative colitis, if adaptby radiation therapy, chemotherapy, and immunotherapy, genetic defects (cystic fibrosis)	
Lactococcus		Mainly non pathogens, and an atypical necrotising pneumonia, necrotic abscess in a middle-aged patient, atypical necrotising pneumonia	EFSA 2012,2013
Leuconostoc	*L. citreum,* *L. lactis,* *L. mesenteroides* *L.pseudomesenteroides*	Mostly non-pathogenic, single cases of infections, in a patient with coexisting rheumatoid arthritis and tuberculosis arthritis and a case of neonatal sepsis and a reportedinfection in a patient who had undergone to liver transplantation	EFSA 2012, 2013
Streptococcus	*S.pyogenes,* *S.pneumoniae,* *S.agalactiae,* *S.mutans,* *S.sobrinus*	No reports of clinical infections since 2011	OECD 2011, EFSA 2012, 2013
Enterococcus	*E. faecalis,* *E. faecium*	Some strains are pathobionts performing hemolytic activity , antibiotic resistance and antibiotic resistant plasmid transition. Not included in QPS list	OECD 2011, EFSA 2012, 2013
Bifidobacterium	*B. longum* *B. infantis*	Mostly non-pathogenic, singlecase reports generallyin immune compromised hosts, septicaemia in an extremely low-birthweight infant	EFSA 2012, 2013
Saccharomyces		Mostly non-pathogenic, single cases of infections, an opportunistic pathogen	EFSA 2012, 2013

2.1.6 Legislation on probiotics for human consumption

Governmental regulations on probiotics is complex. The FAO/WHO guideline constituted the background for each member nation to establish their own regulation. However, such is not always the case for every member nation.Probiotics are subcategorized under different categories and are defined separately by different countries.The following are some of the examples.

United States of America

In the US, safety, labelling and health statements made on conventional foods, medicinal foods, food for special dietary use, and dietary supplements are regulated from the Food and Drug Administration (FDA). Probiotic microorganisms fall into the 'complementary and alternative medicine' category (CAM) that is a combination of old practiced medicine and recent ones (FDA, 2006).In 1992the U.S. Congressauthorized unconventional therapies in officies, namely from; 1992-1997 at the Office of Alternative Medicine (OAM) in order to unravel the unconventional medicinal practicies and, because at that time the 1/3 of Americans as reported was using some forms of CAM products(FDA, 2006), from 1998-today; at theNational Center for Complementary and Alternative Medicine (NCCAM).Moreover, in 2002 the FDA established the Office of Combination Products (OCP) for dividing regulatory responsibilities forfoods, substances within food,foods containing/or products of combining elements, drugs, devices, and biologics inseperate Centers— CFSANCDER, CDRH, and CBER.Confirmation and verification of the claims including the written-tence and order of functional/nutritional claims are authorized by FDA. In 2005 NCCAM updated the term of CAMas 'a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine' (FDA, 2006, Hoffman *et al.,* 2013). That group is subcatecorized in four categories or domains, in which probiotics among others, are regulated under the *Biologically-based practices.*

The manufacuter (petitioner) addresses its intented use taking into account current available claims and evidence available in the application envelope, using methods from the Association of Official Analytical Chemists (AOAC). If no AOAC method is available, the petitioner must include in the submission the assay of the method used and relevant data that establish its validity. Depending on a probiotic product's intended use, FDA might regulate it as dietary supplement, food, a cosmetic, medical device, drug and as a biological product under the Federal Food Drug and Cosmetic Act (the Act) or, if its intendent use is for prevention of a communicable diasease, then it is considered as a biological product under the Public Health Service Act (PHS Act). Considering the categories , non of the applications committed has been accepted from FDA. If a probiotic product is considered as a drug by the Act , then it is considered as a biological product under the PHS Act aswell (FDA, 2006). The incidence of

misleading or "implied claims" and labeling on advertized products is under the control of the Federal Trade Commision (FTC) regulators. The FTC's Enforcement Policy Statement on Food Advertising is determining if an advertisement meets FTC requirements on an advertisement are considered.

A major impact for the rising regulatory demand on probiotic microorganisms in the US may be an outcome of The Human Microbiome Project (HMP) (further information avilable at http://commonfund.nih.gov/hmp/index), as it revealed findings on the importance and role of the human microbial flora in health, in illnes and its contribution in diaseases. As so, it rise the consciousness on the beneficial manipulation towards health, additionally with both, the expansion of their use and the increased imports of such products in the country (Hoffman *et al.,* 2013, 2014).

Claims on how the product affects the structure or function of the body are allowed, but claiming that the product reduces the risk of a disease (health claims) must have FDA's pre-approuval(FDA, 2006,Sanders & Levy 2011, NIH & NCCAM 2012). Therefore, requesting FDA's approval in CAM products for specific treatment of a disease or disorder (a.k.a drugs) need to be proven as safe and effective for its intended use through clinical trials(Sanders, 2008, NIH & NCCAM 2012). Generic requirements for petition are detailed in the US Code of Federal Regulations Title 21.

For a full description on nutritional content claims, U.S. Food & Drug Administration*Claims that Can be Made for Conventional Foods and Dietary Supplements*(Sept. 2003) and for advertisement issues the U.S. Bureau Of Consumer Protection *Dietary Supplements: An Advertising Guide for Industry* (Apr. 2001) (available athttp://business.ftc.gov/documents/bus09-dietary-supplements-advertising-guide-industry) are suggested.

Japan

Japan is one of the few countries that had established an endogenous regulation regarding probiotics before the FAO/WHO guideline was published. In 1984 the Japaneese Ministry of Health and Welfare (MHW) initiated the Food for Specific Health Uses (FOSHU) categoryfor foods and food products that contain functional ingredients. In 1991 the FOSHU system was formally recognized and entered into Health Improvement Act, (Article 26), and Food Sanitation Act, (Article 11), after a predictive reasearch on the rising costs of Japans health care system(Ingvar *et al.,* 2013). Hence, functional foods was first coined in Japan. Probiotics were then included as part of functional food.FOSHU has divided food claims in various categories depending on the level of claim and scientific evidence and has allowed these claims depending on the strength of the evidence and the supporting data.

The government has designated FOSHU health claims into different subcategories as per their health '*use*' in gastrointestinal health, cholesterol moderation, hypertension moderation,

lipid metabolism moderation, sugar absorption moderation, mineral absorption as well as for bone and tooth health. New claims and combination of claims are approved on a regular basis(Tamime, 2005).

The Ministry of Health and Welfare, now the Ministry of Health, Labour and Welfare (MHLW) approves the overseas applications and evaluates them maximum within a year. The process of regular FOSHU registration depends upon the information which must include safety, efficacy, processing, formulation data, analytical method and chemical and physical analysis, as well as other specific information, accompanied by product samples and labels with proposed claims. This whole information must be in a Japanese scientific journalas it is also required. In few words, the approval process for obtaining a licence proceeds as follows. First, the manufacturer compiles scientific data on the health effects, physicochemical properties, appropriated level of intake, safety, nutritional composition, and test methods for the food or compound of interest. The issue of minimum viability in the final product is not an official requirement although several industrial organizations, such as the Japanese Fermented Milks and Lactic Acid Bacteria Beverages Association, are occupied on that field (Lee & Salaminien, 2009). That organization stipulated for example that a product containing more than 10^7 viable bifidobacteria/g or ml is to be considered a probiotic food. Then, the application is submitted to the Ministry of Health and Welfare, with the above information plus descriptive information. The application is evaluated by the Japan Health Food and Nutrition Food Association, and finally by a committee appointed by the Ministry of Health and Welfare, which can approve the application (Balcazar *et al.,* 2006 and references therein, Lee & Salaminien, 2009); Furthure information available athttp://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html).

Europe

Following a series of food crisis in the late 1990s, Europe was second in establishing a definition of functional foods and implementing a regulatory commission on functional foods.

Because of increasing interest in the concept of "Functional Foods" and "Health Claims", the European Union set up a European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) programme coordinated by the International Life Sciences Institute (ILSI) Europe in 1995, followed by four more Framework Programmes (FP) since now.

In the European Union established,in 2002,an agency called European Food Safety Authority (EFSA) (**Fig. 5**), to evaluate, function and provide scientific advice on risks associated with food in the European market (178/2002/EC).

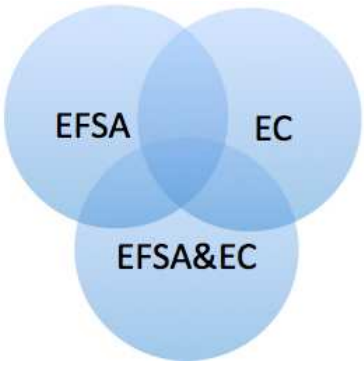

Figure 5: Regulatory scheme.
After the approval from EFSA, following the current regulatory approaches and directives made by the European Council, a final approval from the last regarding claimsare then re-evaluated and hencedessision making is considered to be made by both organizations.

According to EUs regulatory framework all microbial cultures present in food needs to satisfy the legal requirements, as so aprobiotic can be categorized, or regulated and marketed, as food supplement. Both EFSA, and the Commission have made considerable efforts to ensure clarity on the issue of valid health claims; meaning any claim which states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health.Health claims on functional foods are regulated on a national level. Briefly, petitioners are requested to submit their application files at the national competent authority of theMember State, i.e.EFET in Greece, for authorization. Then, if the application is considered valid, it goes forward to the EFSA for validation. Finally, it is has to be authorized by the Commission. EFSA is engaged with stakeholders to outline and clarify the process followed by evaluation of claims from EFSA's Panel on Dietetic Products, Nutrition and Allergy NDA (1924/2006/EC).

Moreover, in 2011,for the improvement of the health claim application procedures, EFSA made available a general guidance document covering the principles in the evaluation process (EFSA 2011a)and an additional specific guidance regarding scientific requirements for health claims related to gut and immune functions (EFSA *Guidance on the scientific requirements for health claims related to gut and immune function, 2011*). From 2012 particular information that further specify the ingredients of a food are mandatory to appear on foods. One of those is the list of ingredients. An ingredient is '*any substance, including additives, used in the manufacture or preparation of a foodstuff and still present in the finished product, even if in altered form*' shall be designated in that list by their specific name. Accordingly, any microorganisms used in the manufacture of foods should appear therein. This includes probiotics (1169/2011/EC).

Although the EU has invested over EUR 70 million in research, more than 422 applications submitted under the Health Claims Regulation, regarding probiotic claims, they have not received a positive assessment by EFSA or approval from the Commission, making

probiotics one of the topics that have been most negatively affected by the Regulation. The current settled rules for pettitioners can been found in 907/2013/EC. EU legislation is dynamic. It is therefore advised to consult home pages of legislative authorities in order to obtain the latest information (**Table 3**).

Table 3. Institutes for Further Information on Health Claim Legislation in the EU.

Source: Extracted from Lee & Salaminien, 2009

Institutes	Country	Acronym	Homepage
European Food Safety Authority	EU	EFSA	http://www.efsa.europa.eu/en.html
Ministry of Health	Belgium		www.health.fgov.be/
Elintarvikevirasto	Finland	EVIRA	http://www.evira.fi/
Agence Française de Sécurité Sanitaire des Aliments	France	AFSSA	http://www.afssa.fr/
Direction Générale de la Concurrence, de la Consommation et de la Repression des Fraude	France	DGCCRF	http://www.finances.gouv.fr/DGCCRF/
Food Safety Authority of Ireland	Ireland	FSAI	http://www.fsai.ie/
Ministry of Health	Netherlands		www.minvws.nl
Agencia Española de Seguridad Alimentaria y Nutricion	Spain	AESA	www.aesa.msc.es
Swedish Nutrition Foundation	Sweden	SNF	http://www.snf.ideon.se/
Food Standards Agency	UK		http://www.food.gov.uk/foodlabelling/ull/claims/

2.1.7 Economic facts, Current Status and Objectives

Regulatory changes in the area of health claims have a strong impact on market performance. The market of probiotics has grown significantly in the past years due to the popularity of probiotic yogurts. In addition wth the rise of technology and consumer awareness, probiotics are also moving increasingly beyond the dairy sector, providing that regulatory hurdles can be overcome. Probiotics are at the cutting edge of food sector innovation, and the sector is growing by a rate of around 6% each year.

The market survey of probiotic-related products varies between countries. In 2007,the EU commissioned thestudyof SANCO "*The use of substances with nutritional or physiological effect other than vitamins and minerals in food supplements*" at Six different categories were included:

- amino acids
- enzymes
- pre- and probiotics
- essential fatty acids
- botanicals and botanical extracts
- miscellaneous bioactive substances

A total of31 substances were selected, although the total number is estimated to be over 400, as the main source of data used in order to review the EU market. The statistics were provided from the specialist company Euromonitor International (hereafter Euromonitor). More specifically, the market size of probiotics among the other categories was consisting of other substances available the market 44% in Italy, 13% of the market in Belgium, 3% in Poland and a poor 1% in the UK and Germany. For Greece, there is no documentation for probiotics but there is a 9% of the market in yeast products like *S.bulardii.*In that reportprobiotics in supplementary form seem to consist one of the most commercially important substances being sold in 2005, as a single form product and/or formula, holding EUR 252.4 million across 17 EU Member States(**Fig. 6**) (SANCO, 2007, European Commission Staff Working Document on*Characteristics and perspectives of the market for food supplements containings substances other than vitamins minerals* of 2008).

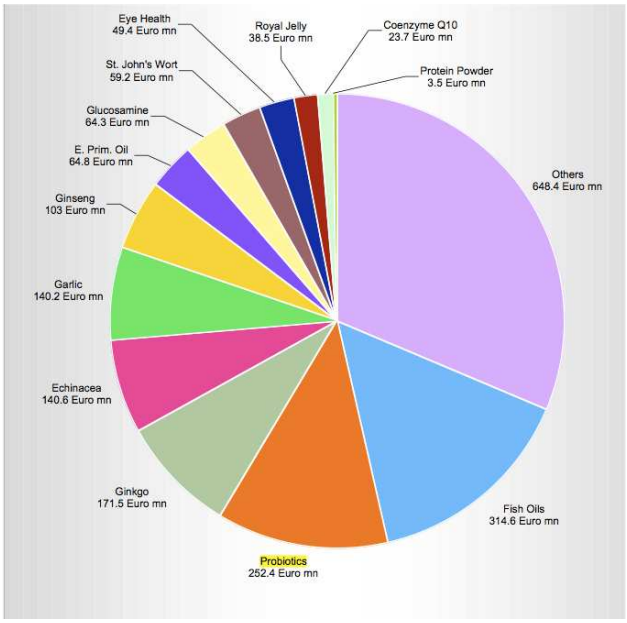

Figure 6. Market size of probiotics among other food supplements containing substances other than vitamins and minerals
(Source: Extracted from Euromonitor 2005inSANCO, 2007)

Although the probiotic industry is suffering from the impact of the ban onprobiotic in the EU, in 2013, over 60% of functional food products are directed towards digestive health, with prebiotics and probiotics being the most common worldwide (Binns, 2013). Moreover, in 2015 a surprisingly fast growth of probiotic yoghurt consumption was recordedfor China, India and Japan. The US market undergoes significant changes with probiotic supplements continuing to grow, but yoghurt recorded the first year of decline. Consumer preferences regarding consumption of probiotics seems to move from yoghurt towards supplements. Transparency regarding the dosage, convenience and the attached *zero calories* tag play a key part in consumer choise. Thus, in 2015 probiotic supplements was the fastest growing dietary supplement type globally, rising by 8% in contrast with the global sales of probiotic yoghurt, that grew by 6% the same year.

The EU's probiotic claims ban is estimated to contribute to a revenue loss for probiotic yoghurt of around €2.5 billion, between 2012 and 2020. Between 2015 and 2020 this market stands to lose a further EUR1 billion in retail value as result of the ban of probiotic claims, whilist China dominate growth in probiotic yoghurt, with an estimated increase of US$9.9 billion and US are set to rise by US$1 billion.

Despite the lack of approved health claims beyond these broadly relating to digestion, probiotics undergo numerous studies and clinical trials, opening up further areas for development, including infant health and antimicrobial resistance. With digestive discomfort of key concern for the target group, there is also a clear gap in the market for "free-from"

probiotic products. Interestingly, according to research reports of Euromonitor the spread of chronic diseases, coupled with a sharp rise in the elderly population, will create a leverage point for a new generation of probiotics to be developed, mainly probiotic fermended milksespecially for the area of cardiovascular health and diabetes.(The information above were extracted from *global market research reports* of Euromonitor International; more information available online at http://www.euromonitor.com).

Framework Programes (FP)dating back from 1998 and funded by the European Union are trying to unravel the abilities of the probiotic innovative marketby amending projects and research proposals within the community on the probiotic field among others, and are still happening. Those programmes and information on the projects running are available at http://cordis.europa.eu.

2.2 Section II:Prebiotics

2.2.1. Definition

Prebiotics are consisting an additional strategy that is able to manipulate the intestignal microbiota.Selectively fermended ingrediends have the potential of modifing both the composition of comencial microflora and the colonization of the probiotic strains as well as the activity of the aformentiond in a way that is beneficial for hosts health.

The term prebiotic was first coined in the mid-1990s by Gibson and Roberfroid, and the first definition was a 'non-digestible food or feed 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'(Gibson & Roberfroid, 1995, Gibson *et al.,* 2010, IRTA, 2015). This definition was updated in 2004, as 'selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the GI microflora that confer benefits upon host wellbeing and health' (Gibson *et al.,* 2004; 2010). The status of prebiotics is not established on an international basis. The term prebiotic must be used only when a health benefit related to modulation of the target site microbiota has been demonstrated in the target host (FAO/AGNS, 2007). Thus, a prebiotic is defined as *a non-viable food component that confers a health benefit on the host associated with modulation of the microbiota*(FAO/AGNS, 2007). The most recent was agreed at the 2010 Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) "A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health." (Binns, 2013)

2.2.2 History of prebiotics

Japanese researchers in the years between early 70's and late 80's,were the first who recognised the value of non-digestible oligosaccharides, initially from animal feed where their addition to the feed of piglets helped to relieve and prevent scouring (diarrhea)(Binns, 2013). Japanese researchers also recognised the value of oligosaccharides in human milk and later demonstrated that consumption of fructo-oligosaccharides and galacto-oligosaccharides led to an increase in intestinal bifidobacteria and stimulated their growth in the human gut (Yazawa & Tamura, 1982 inTamime 2005,Binns, 2013). However, it wasn't until 1995 that the scientific concept for human gut microbiota modulation by prebiotics was introduced. The term "prebiotic" is coined to Gibson and Roberfroidwho linked the concepts of prebiotics and probiotics for promoting beneficial populations of intestinal bacteria(Gibson & Roberfroid,

1995). Since then, a wealth of research information has been accumulated.

2.2.3 Prebiotic Characteristics

In order for a dietary substrate to be classed as a prebiotic, at least three criteria are required: (1) the substrate must not be hydrolysed or absorbed in the stomach or small intestine, (2) it must be selective for beneficial commensal bacteria in the large intestine such as the bifidobacteria, (3) fermentation of the substrate should induce beneficial luminal / systemic effects within the host (Manning & Gibson, 2004; IRTA,2015).

In 2007 the American organizations of Food Quality and Standards Service (AGNS) and the Food and Agriculture Organization of the United Nations (FAO) prepared a putativeguidelinewith criteria, and methodologiesfor conducting a systematic approach for the evaluation of prebiotics leading to their safe and efficacious use in food.

In order to characterize a product, or an ingredient of a product, as prebiotic proper characterization according to AGNS and FAO has to be carried out as followed:

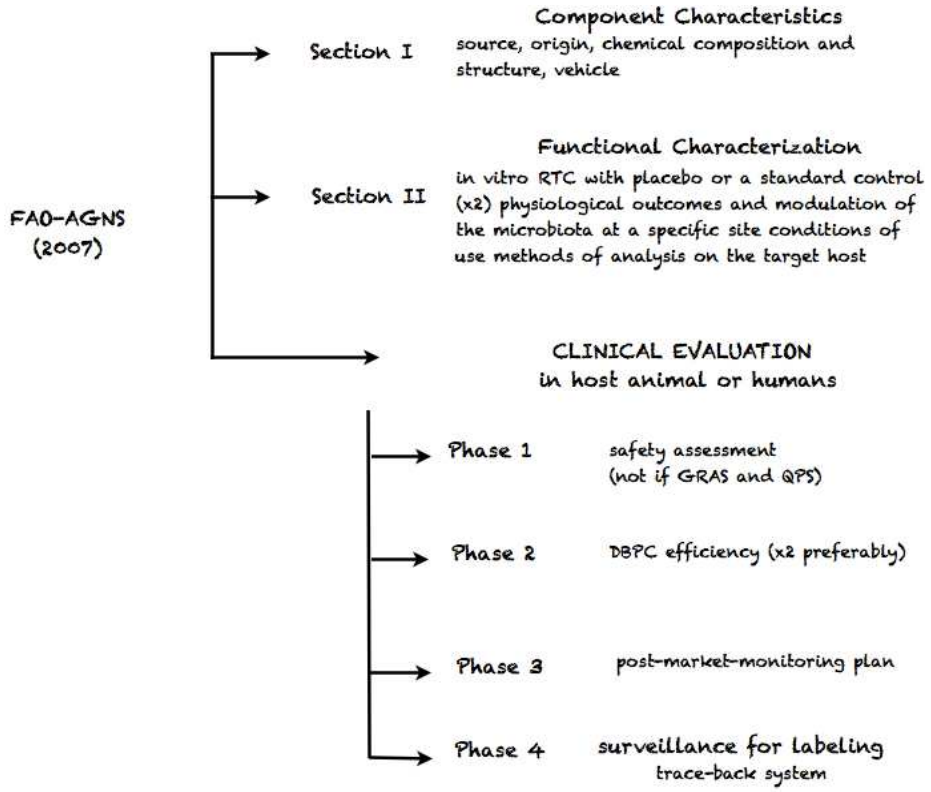

Figure 7. FAO/AGNS recommendated evaluation process of 2007.
Reccomended phases for the evaluation and substaination of a potential prebiotic for a targeted host. Phase 1, is not required ifthe product has a history of safe use in the target host.

Further analysis of each phase is presented below;

Section I: Component Characterization;information about the source, origin, chemical composition and chemical structure, along withnutritional recommendations have to be carried out firstly. The potential prebiotic has to be in a pure form.

Section II: Functional Characterization; in vitroandin vivoevaluation of prebiotic effectiveness using targeted host conditions and laboratory animals in orderto correlate a specific function at a specific site with the physiological effect and its associated timeframe. Delivery form, concentration and amount, in which it is ment to be delivered to the host can be desided.

Section III: Clinical Evaluation. The Clinical Evaluation Prossesss is divided further in;

Phase I: Safety assessment;in vitroor in vivo in target animals or Phase 1 clinical study in humans. toxicological studies to evaluate safetyare not required if, according to local legislation, the product has a history of safe use in the target host. Suitably sized randomized control trial (compared to placebo or a standard control substance) is required, preferably with a second independent study

Phase 2: Double Blind Randomized Clinical Trial;in order to correlate the measurable physiological outcomes at a specific site (primarily the gastrointestinal tract, but potentially also other sites such as vagina and skin). Based upon current knowledge, the prebiotic should not alter the microbiota in such a way as to have long term detrimental effects on the host. Preferable a Second Independent RTC

Phase 3: The Technical Meeting recommends that prebiotic producers, medical professionals and public health officers consider some form of system to monitor the health outcomes of long- term prebiotic administration.

Phase 4: This is suggested as a means to gain insight into potential side effects as well as assess long-term benefits. A necessary prerequisite for surveillance is a proper trace-back system.

The choosen biomarkers of clinical endpoints may be satiety (measured towards carbohydrates, fats, total energy intake); endocrine mechanisms regulating food intake and energy usage in the body; effects on absorption of nutrients (e.g. calcium, magnesium, trace elements, protein); reduced incidence or duration of infection; blood lipid and classic endocrine parameters; bowel movement and regularity; markers for cancer risk; changes in innate and acquired immunity that are evidence of abenefit to health.

Qualifications of a substrate to be prebiotics are:

a. Bifidogenic effects are not sufficient without demonstrated physiological health benefits.

b. It is recognized that at this time, determining events that take place within compartments of the intestine are often difficult. A specific site sampling or more sophisticated methods(in example fecal screening for bifidobacteria) can reliably link the microbiota modulation with health benefits.

Disadvantages;Prebiotics are not as potent as antibiotics in eliminating specific pathogens. A main disadvantage of prebiotics is that overconsumption can cause intestinal bloating, pain, flatulence, or diarrhea. Moreover if consumed during active diarrheaside effects of simple sugar malabsorption may exacerbate.

2.2.4 Prebiotic Mechanism of action

Prebiotics are characterized by their nondigestible or partially digested and nonabsorbable in the small intestine abilities (Slavin, 2013). Besides, prebiotics can be fermented by purportedly beneficial bacteria in the gut and mouth, and in a lesser extent by potentially pathogenic bacteria (de Vrese & Schrezenmeir, 2008). Most prebiotics can be found naturally in various foods. For example, most nondigestible oligosaccharides are natural components of many common foods including honey, milk, and various fruits and vegetables in low concentrations mainly (Lee & Salaminien, 2009).

The chemical composition and structure of the prebiotic can be expected to determine physiological effects and which microbial species are able to utilize it as a carbon for energy source in the bowel (Slavin, 2013). However, despite the diversity in molecular sizes, sugar compositions, and structural linkages within the range of prebiotic carbohydrates, it is the bifidobacteria that are almost universally observed to respond (Slavin, 2013).

To date, administration of prebiotics selectively modifies the composition of the intestinal microbiota through several mechanisms and favors the saccharolytic fermentation resulting in increased production of short chain fatty acids (SCFA) (Slavin, 2013). These SCFA play a pivotal role in the health benefits associated with prebiotic intake as they acidify the colonic lumen, which influences metabolic pathways and inhibits pathogens, and acts as signaling molecules on specific receptors (Sun & Riordan, 2014).

As addressed from The Prebiotics Task Force of ILSI Europe the health functions of prebiotics are addressed to the following areas: modulation of gut microbiota composition and activity, immunity, inflammation, mineral absorption, colon cancer, energy homeostasis, satiety regulation and body weight gain (Roberfroid *et al.,* 2010, Binns, 2013). Prebiotics enhance the growth of the endogenous microbiota or possibly stimulate the growth of probiotics if present (Roberfroid *et al.,* 2010, Binns, 2013, Ghouri *et al.,* 2014). Thus, probiotics and prebiotics share many common mechanisms of action mediated through an impact of microbes on the host (Binns, 2013). In the case of health effects that relate only to prebiotics, the mechanisms are less well known (Binns, 2013).

Over the last decade, data has convincingly demonstrated that particular prebiotic food products/ingredients/supplements can, upon oral consumption, selectively modulate the gut microbiota composition and possibly its activities (FAO/AGNS, 2007, Roberfroid *et al.,* 2010). Some, but not all, studies have also reported a reduction in the concentration of pathogenic bacteria, such as clostridia and *Salmonella* (EFSA, 2010). The more accumulating the data are, the more it will be recognised that such changes in the composition of the fecal microbiota, especially increase in bifidobacteria can be regarded by many as a marker of intestinal health (FAO/AGNS, 2007, EFSA, 2010). Specific beneficial health effects have been reported on dietary consumption of specific prebiotic food products, ingredients and supplements, which are relevant for infants as well as for adults in both healthy and compromised status, respectively (Roberfroid *et al.,* 2010).

2.2.5 Types of prebiotics

Most identified prebiotics are carbohydrates. Within these, there is a wide diversity of molecular structures. However, these carbohydrates share a number of physiological traits important to their beneficial effects(Lee & Salaminien 2009, Slavin 2013, Ghouri *et al.,* 2014). To date, the largest number of reported studies and the most consistent evidence accumulated for prebiotic effects have been for several non-digestible oligosaccharides (NDOs). These include fructooligosaccharides (FOS), and the polyfructan inulin, galactooligosaccharides (GOS) and lactulose. A number of other NDOs, to which less rigorous study has been so far applied, have at least indications of prebiotic potential. These include lactosucrose, xylo- (XOS), isomalto- (IMO), and soybean- (SOS) oligosaccharides (Burns & Rowland 2000, Gaggìa *et al.,* 2010; Calafiore *et al*., 2012,Slavin 2013, Preidis & Versalovic 2014, Conlon & Bird 2015). Indeed, it appears that a wide range of NDOs can stimulate the growth of bifidobacteria and new potential prebiotics continue to emerge. In vitro and animal feeding trial data showing potential bifidogenic effects have been reported for gluco- and galactomannan oligosaccharides, alpha-glucooligosaccharides, pectic-oligosaccharides, gentiooligosaccharides, and oligosaccharides from agarose among others (Lee & Salaminien 2009, and references therein). There are evidence that some polysaccharide dietary fibers, such as resistant starches, xylooligosaccharides from arabinoxylan, resistant dextrinsand plant gums have prebiotic potential is accumulating, but to date remains limited largely to in vitro and animal studies (Lee & Salaminien 2009, Śliżewska *et al.,* 2012). It is well established that lactulose, short-chain oligosaccharides, inulin, resistant starch, and dietary fiber are not toxic, even at high doses (Slavin, 2013).

According the term of prebiotics', as FAO and AGNS described in 2007, the administration of probiotics combined with prebiotics may provide definite health benefits to the host by synergistic action. Understanding how prebiotics perform their positive effects, is

an issue of debate among scientists nowadays with the most substances consisting to be carbohydrates (Hutkins *et al.,* 2016).

2.2.6 Production of prebiotics

The existing definitions of a prebiotic, as stated above, while differentiating this class of non- digestible food ingredient within the dietary fibres and broadly serving the more common and well studied prebiotics. The main approaches used for the production of prebiotic carbohydrates are reviewed below in **Table 4** as well as a typical production process for nondigestible oligosaccharides is shown in **Figure 7.**

Table 4.Main approaches for production of prebiotic carbohydrates.
Sorce: Extracted from Lee & Salaminien, 2009

Approach	Process	Prebiotic Examples
Direct extraction	Extraction from raw plant materials	Soybean oligosaccharides from soybean whey Inulin from chicory Resistant starch from maize
Controlled hydrolysis	Controlled enzymatic hydrolysis of polysaccharides; may be followed chromatography to purify the prebiotics	Fructooligosaccharides from inulin Xylooligosaccharides from arabinoxylan
Transglycosylation	Enzymatic process to build up oligosaccharides from disaccharides; may be followed by chromatography to purify the prebiotics	Galactooligosaccharides from lactose Fructooligosaccharides from sucrose Lactosucrose from lactose + sucrose
Chemical processes	Catalytic conversion of carbohydrates	Lactulose from alkaline isomerization of lactose Lactitol from hydrogenation of lactose

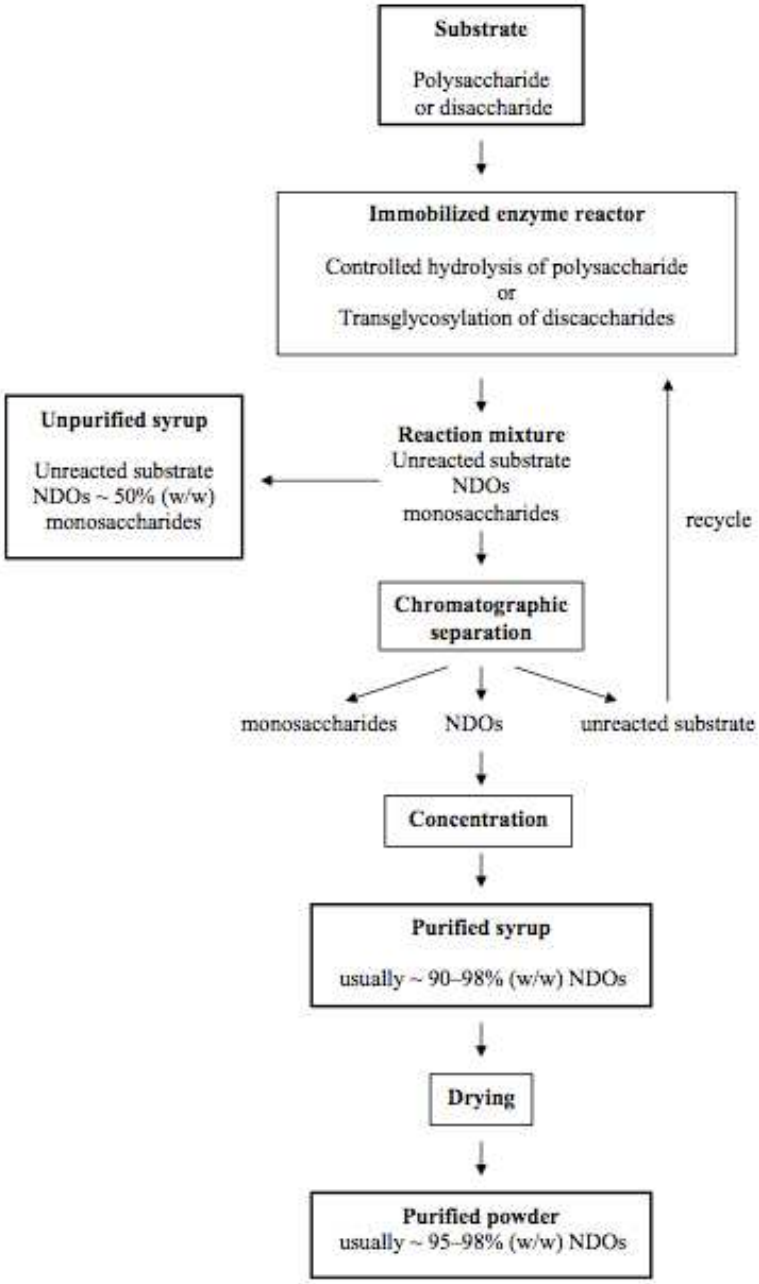

Figure 8. Production flowchart for the manufacture of prebiotic oligosaccharides.
Sorce:Extracted from Lee & Salaminien, 2009

Food-grade oligosaccharides are not pure products, but mixtures containing oligosaccharides of different degrees of polymerization (dp), the parent polysaccharide or disaccharide, and monomer sugars (Śliżewska *et al.,* 2012). An example of a typical product mixture produced by transfructosylation of sucrose is shown in **Figure 8.**

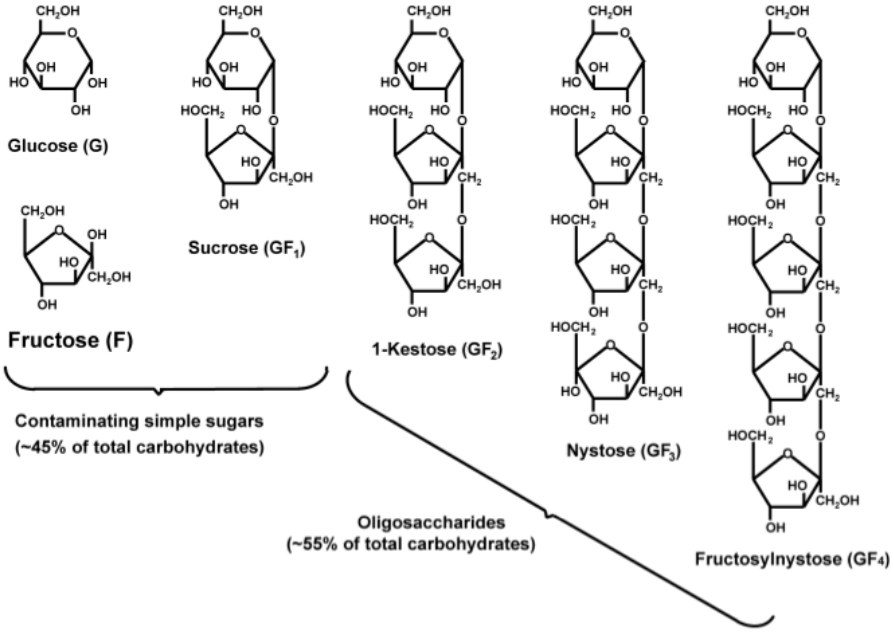

Figure 9. Commercial fructooligosaccharide production via transfructosylation of sucrose. Following the enzymatic reaction, the reaction mixture contains 50–60% oligosaccharides.
Sorce: Extracted from Lee & Salaminien, 2009

Chromatographic processes are used to remove monosaccharides and unreacted sucrose to produce higher purity oligosaccharides.

Oligosaccharide products are sold at this level of purity, often as syrups. Chromatographic purification processes are used to remove contaminating mono- and disaccharides to produce higher purity oligosaccharide products containing between 85 and 99% oligosaccharides, which are often dried to powders (Śliżewska *et al.,* 2012). Different manufacturing processes also produce slightly different oligosaccharide mixtures. For example, FOS mixtures produced by transfructosylation of sucrose contain oligosaccharides between three and five monomer units, with the proportion of each oligosaccharide decreasing with increasing molecular size (Tamime, 2005, Gibson *et al.,* 2010, Śliżewska *et al.,* 2012). These oligosaccharides contain a terminal glucose with b1-2 linked fructose moieties. In comparison, FOS produced by the controlled hydrolysis of inulin contain a wider range of b 1-2 fructooligosaccharide sizes (dp 2–9), relatively few of which possess a terminal glucose residue (Tamime, 2005, Śliżewska *et al.,* 2012). Even different b-galactosidases used in the production of GOS will produce oligosaccharide mixtures with different proportions of b1-4 and b1-6 linkages (Meyer *et al.,* 2015). Hence, there can be some diversity between the structures of oligosaccharides produced by different manufacturers. The precise impact of these differences in health have to be determined. Some differences have been observed in the ability of lactobacilli and bifidobacteria to use oligosaccharides of different degrees of polymerization which has also translated to different bifidogenic potencies *in vivo* (Lee & Salaminien 2009, and references therein).

2.2.7 Positive effects

Prebiotics have physicochemical and organoleptic properties that make them useful food ingredients. For example, NDOs are sweet and are used as low-cariogenic and low-calorific sugar substitutes, while polysaccharides such as inulin are used as fat replacers. Furthermore, NDOs can be used to increase viscosity, but the effect on Malliard reactions - the increased susceptibility of proteins to heat damage in the presence of various carbohydrates (McDonald *et al.,* 2010)- is not clear (Lee & Salaminien, 2009, Śliżewska *et al.,* 2012, Meyer *et al*., 2015), alter water retention, depress freezing points, suppress crystal formation (de Vrese & Schrezenmeir, 2008, Lee & Salaminien, 2009) and colors of food (de Vrese & Schrezenmeir, 2008). Hence, they are used commercially in a wide variety of foods and beverages.

Moreover,as regards the association between prebiotics and the strength of the bifidogenic effect, the cell counts of bifidobacteria after prebiotic ingestion depend mainly on the actual number of bifidobacteria in the host (de Vrese & Schrezenmeir, 2008). It is really difficult yet to address the beneficial effects on prebiotics alone, without taking into account the growth of the endogenous microbiota (Binns, 2013).

As so, the potential effects of prebiotics on health can be addresed on digestion, absorption, immunity, brain/neuronal function, or appetite regulation. A number of largely prophylactic health targets have been proposed for prebiotics. As might be expected, these overlap considerably with the targets of probiotic interventions. Some effects have therapeutic value for specific disorders while others are potentially beneficial to the population at large. Hence, prebiotics have found applications both as pharmaceuticals and as functional food ingredients. Below some examples are being presented;

Chronic constipation

Lactulose is widely used as a pharmaceutical to treat constipation. It has proven efficacy in a number of placebo-controlled trials at doses between 10 and 20 g/day (Banares, 2006,Quah *et al.*, 2006) even in patients with chronic constipation. The effect is not caused by modifications to the composition of the intestinal microbiota. Lactulose has an osmotic effect, since it is a relatively small molecule that is not digested or absorbed, trapping fluid, accelerating transit in the small bowel, and increasing ileocecal flow. Its rapid fermentation to SCFA and hydrogen also contributes to this effect and induces peristalsis (Jouet *et al.,* 2006). A number of other NDOs, such as inulin has been shown to mildly improve stool frequency and consistency in adults (Lee & Salaminien 2009, and references therein) although their applications are targeted towards functional foods rather than pharmaceutical applications.

Hepatic Encephalopathy

Lactulose (and lactitol) are also front-line therapeutic agents for the treatment of hepatic encephalopathy (HE). This neuropsychiatric condition results from liver dysfunction caused by cirrhosis or hepatitis. It includes a spectrum of symptoms ranging from subtle changes in cognition and personality to lethargy, stupor, and coma (Dbouk & McGuire, 2006). A dysfunctional liver is unable to clear ammonia from the blood stream, which then accumulates to levels toxic to the central nervous system. The ammonia is produced by the intestinal microbiota as an end product of protein metabolism, and ammonia readily crosses the intestinal epithelium to enter circulation. Lactulose and lactitol act by limiting both ammonia production by the microbiota and the absorption of ammonia from the intestinal lumen (Dbouk & McGuire, 2006). Acidification of the colonic lumen resulting from SCFA production inhibits urease positive and deaminating bacteria (implicated in intestinal ammonia production) and importantly leads to the protonation of ammonia to ammonium ions in the intestinal lumen. Ammonium ions cannot readily cross the intestinal epithelium, and so the drop in pH effectively traps ammonia in the lumen. Lactulose and lactitol have similar efficacy, although lactitol is more palatable and produces more rapid results with fewer side effects (Dbouk & McGuire, 2006). However, to be effective lactulose and lactitol are delivered in high doses (30–60 g/day). This of course has a large laxative effect, causing significant discomfort. Therefore, there is an interest in using larger NDOs and soluble fibers that ferment rapidly, but with less osmotic effect in the gut.

Amelioration of Inflammatory Bowel Disease

The precise etiology of IBD remains unknown. However, there is accumulating evidence that a genetic predisposition to develop an overzealous inflammatory immune response to components of the intestinal microbiota is responsible. Prebiotic and synbiotic interventions have ameliorated colitis in different rodent models of IBD. Studies in both animal models and human subjects have shown that prebiotic-induced stimulation of Bifidobacterium numbers has been associated with downregulation of inflammatory markers in intestinal mucosa (Hoentjen *et al.,* 2005, Lara-Villoslada *et al.,* 2006; Lee & Salaminien 2009) and evidence of increased immune regulation (Hoentjen *et al.,* 2005, Lindsay *et al.,* 2006; Lee & Salaminien 2009).

Prevention of Infections

Prebiotic oligosaccharides may provide protection against enteric infections through competitive inhibition of pathogen adherence to the mucosa. Many intestinal pathogens, such as *Escherichia coli, Salmonellae* and *Campylobacters* utilize oligo-saccharide receptor sites in the gut for attachment (Gibson, McCartney & Rastall, 2005). NDOs can act as structural mimics of the pathogen binding sites and act as soluble decoys. Human milk oligosaccharides act in this way to block the initial binding of a range of pathogens to inhibit colonization (Gibson, McCartney & Rastall, 2005, Shoaf *et al.,* 2006). Lactulose consumption at high dose (up to 60

g/day) is effective in eliminating salmonella from the intestinal tract of chronic human carriers and it is used as a pharmaceutical for this purpose in some countries (Schumann, 2002; Lee & Salaminien 2009).

Mineral Absorption

Non-digestible, fermentable carbohydrates in general, a number of prebiotics have been shown to increase mineral absorption, but the precise mechanisms of prebiotic-mediated improvements in mineral uptake remains unclear. Calcium and magnesium are the main minerals for which uptake is improved. Moreover, increased calcium absorption in the colon stimulated by prebiotics has further been demonstrated to improve markers of bone health in humans (Holloway *et al.,* 2007, Abrams *et al.,* 2005). Animal and human studies comparing prebiotics of differing chain lengths, fermentation patterns, and doses have shown that higher doses and more persistent fermentation profiles are more effective (Lee & Salaminien 2009, and references therein).

Prevention of Colorectal Cancer

There are little epidemiological data available for the use of prebiotics in cancer prevention but there are several studies reporting protection by prebiotics against the development of preneoplastic lesions and tumors in rodent models of colon carcinogenesis.

Weight Management and Improving Insulin Sensitivity. Since NDOs are sweet and not digested, they have a low calorific value and are used as low energy, low glycaemic index sweeteners that are also suitable for individuals with diabetes. Preliminary data also suggests that SCFA production resulting from prebiotic fermentation (and in particular acetate) could improve insulin sensitivity (Lee & Salaminien 2009, and references therein).

The composition of the human intestinal microbiota changes naturally with age, and in early infancy the microbiota is believed to be particularly important in correct functioning of the gut and maturation of the immune system. Further examples of the prebiotic effect in different life stages can be found at Lee & Salaminien 2009.

2.2.8 Legislation

The regulatory regimes for nondigestible carbohydrates have been under active review in many countries in recent years.

In 2009, according to FAO and Codex Alimentarius, carbohydrate polymers, with 10 or more monomeric units, which are not hydrolysed by the endogenous enzymes in the small intestine of humans, hence prebiotics, are recognized as Dietary Fibers (DF) (Codex Alimentarius Guidelines on Nutrition Labelling. Last revised, 2013). According to the definition, it can belong to two categories of carbohydrate polymers, those which have been obtained from food raw material by physical, enzymatic or chemical means and which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities, or at the synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health as demonstrated by generally accepted scientific evidence to competent authorities. This has allowed recognition of these products as having some health benefits on product labels. The use of GOS and FOS as ingredients in infant milk formulas has been the subject of intensive regulatory inquiry and its acceptance varies among countries.

As regards EU nations on prebiotic health claims, The European Food Safety Authority (EFSA) has issued scientific opinion on the substantiation of health claims for the following prebiotics: wheat dextrin (2010), fructoolisaccharides, galactoolisaccharides, and polydextrose (2011). The European Commission in Council Directive on nutrition labelling for foodstuffs as regards recommended daily allowances, energy conversion factors and definitions to include dietary fiber (2008/100/EC). Moreover for something to be claimed as prebiotic must comply with the requirements of the regulation on nutrition and health claims (1924/2006/EC).

The EFSA has issued scientific opinion on the *substantiation of health claims related to various food(s)/food constituents(s) and increasing numbers of gastro-intestinal microorganisms, and decreasing potentially pathogenic gastro-intestinal microorganisms.* including prebiotics and probiotics(EFSA, 2010).

Revised Codex standards were released in November 2006 (Codex ALINORM 07/30/26). Readers are referred to the Codex website for current standards (CODEX STAN 72–1981; and 156–1987; www.codexalimentarius.net).Policy in third counties can be found at:http://www.ift.org/knowledge-center/focus-areas/food-health-and-nutrition/prebiotics/policy-and-regulatory-developments-related-to-prebiotics.aspx

Chapter 3: Probiotics in Animal Health

3.1 Section I: Animal Probiotics

3.1.1Deffinition

The word probiotic in relation to microbial feed supplements dates back from 1974, as Parker defined and further discussed in 'Chapter 2 subsection 1.3'.

Fuller in an attempt to improve the definition of probiotics that he had made in 1992, as "mono- or mixed cultures of living microorganisms that beneficially affect the host by improving the properties of the indigenous microbiota" in 1989 redefined it as 'a live microbial feed supplement which benefits the host animal by improving its intestinal microbial balance'. Yoon and Stern in 1995 proposed the definition according to which 'Probiotics are composed of live and beneficial microbes which may colonize the hosts GI tract, resulting on health improvement and maximaze production''.

Even if there is a number of definitions of the term 'probiotic' during the last decades, none of them fits exactly with aquaculture applications. When looking for probiotics intended for an aquatic usage it is important to consider certain influencing factors that are fundamentally different from terrestrial based probiotics.

Probiotic bacteria and probiotic yeasts for animal consumptionare defined and authorized as legally feed additives and clasified as zootechnical additives in the functional groups of gut flora stabilizersin the European Union(Bernardeau & Vernoux, 2013)hence there is no mention of the "probiotic(s)" term in feed (1831/2003/EC).

Feed additives are defined as*"substances, microorganisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the following functions"*(1831/2003/EC, 183/2005/EC; 2011/25/EU):

- Favourably affect the characteristics of feed;
- Favourably affect the characteristics of animal products;
- Favourably affect the colour of ornamental fish and birds;
- Satisfy the nutritional needs of animals;
- Favourably affect the environmental consequences of animal production;

- Favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feeding stuffs; or
- Have a coccidiostatic or histomonostatic effect

According to IFIFjurisdiction comparison document of 2013 probiotics fit best within the zootechnical additives as there are explicit defined functions of those:"digestibility enhancers", "gut flora stabilizer", and "other zootechnical additives"(1831/2003/EC) for example the Direct Feed Microbial (DFMs). DFM is an additive for stabilising the microbial communities of the digestive tract in monogastrics and ruminants (IRTA,2015).

According to the European legislation terminology as found in the European guide to good practice feed materials of 2014, *feed additives are considered products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin, or to improve the animals' performance and health, for example by providing enhanced digestibility of the feed materials* (EFISC, 2014).

In fact, nowadays, there is no standing definition of probiotics that is agreed upon by the majority of probiotics researchers in aquaculture although the most recent one defines them as 'live or dead, or even a component of the microorganism that act under different modes of action in conferring beneficial effects to the host or to its environment'. Hence, it is important in the near future to develop a firm definition of probiotics in an aquaculture point of view to eliminate ambiguity on the term being used.

Aquatic animals have a much closer relationship with their external environment (Kesarcodi-watson *et al.,* 2008). This intensive interaction between the environment and the farmed aquatic animals implies that the definition of probiotics has to be adapted for aquaculture. Based on this statement, a new definition for probiotics has been proposed: 'a live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment' (Verschuere *et al.,* 2000). Apart from the requirement of the probiotic to be a live culture, this definition is a lengthy way of describing a probiotic as 'an entire or component(s) of a microorganism that is beneficial to the health of the host', definition concept that was also suggested two years later for probiotics in aquaculture.

3.1.2 History of probiotic use on animals

The history of live microbial feed supplements goes back thousands of years before their

intended use for human subjects.Over the years, probiotics have been used in a number of different ways in livestock, but in the 1960s it was demonstrated for the first time that *Lactobacillus* strains were able to improve the growth performance of pigs (Agazzi *et al*.,2015).

One of the most convincing demonstrations of the role of the gut microflora in resistance to diseases was provided by Collins and Carter (1978) (Ezema, 2013). They showed that the germ-free guinea-pig was killed by 10 cells of *Salmonella enteritidis* but it required 10^9 cells to kill a conventional grade animal with a complete gut microflora. Animals have in their intestineal population of microorganisms that protects them against diseases.

To our knowledge, the first empirical application of probiotics in aquaculture (Kozasa, 1986) is relatively recent and was designed considering the benefits exerted by the use of probiotics firstly on poultry.As regards pollinatorsMáchová and her colleagues (1997) were the first who added probiotics, without specifying the microorganisms used though, into sugar syrup in order to feed honey bees (*Apis mellifera*) and noticed that this improved bee survival.

Public misgivings on the side effects of the use of antibiotics as growth promoters and as therapeutic agents, amending the ban of them in the EU since 2006 (1831/2003/EC), demanded for effective alternatives, were probiotics could fill the gap. Currently, the legislation prohibits the use of probiotics, among others, as an alternative to antibiotics, in functioning as a controlling or stabilizing influence on the flora of the gut (Ezema 2013 and references therein). The possibility to use feed supplements to achieve better animal health, welfare and productivity through manipulation of the GI tract microbial ecosystem has gained considerable attention for all farm animals including aquatic ones (Chaucheyras-Durand & Durand, 2010). It is believed that gut bacteria have requirements for specific nutrients that may not be adequately provided by the animal's diet. Therefore, feeding these may promote the growth of gut bacteria, thereby improving the microbial profile in the gut. The use of probiotic supplements seeks to improve absorbance of micronutrients in feed (Yirga, 2015), restore diet, antibacterial drugs and stress deficiencies in the gut microflora.

Presently, there is an increasing interest concerning the use of probiotics in the livestock industry (Yirga 2015).

3.1.3. Properties of microbial feed additives

After long term studies, recommended criteria and further details for the evaluation of the probiotic potential on microbial feed additives are heldand designed from the European Association on Feed Additives and Premixtures (FEFANA), which includes the French 'FEEDAP'platform, called SYNPA. Certain guidelines on the evaluation of probiotics in feed guidelines are dating back to 2001, written by EFSA Feedap's predecessor for the Scientific Committee on Animal Nutrition (SCAN).

If the strain is not addresd in theQualifies perception of safety (QPS) listthe name and taxonomic classification of each micro-organism shall be provided according to the latest published information of the International Codes of Nomenclature (ICN), then is considered as a new feed addititve. For a new feed additive the recommended details, criteria and requirements for the evaluation prossess can be found at 429/2008/EC. The three recommended sections that the European registration dossier for future approuvalshould include, for viable strains or for mixtures of them, to be approved and marketed for future use as feed/ zootechnical additives, a detailed safety, efficancyassessments and a post-market monitoring plan (1831/2003/EC; 429/2008/EC; 1924/2006; European Food Safety Authority 2011;FEEDAP 2012; IFIF 2013)

A summary of the proposed framework for the above,adopted from the Guidance for the preparation of dossiers for zootechnical additives of EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) in 2012, can be adressed as follows.

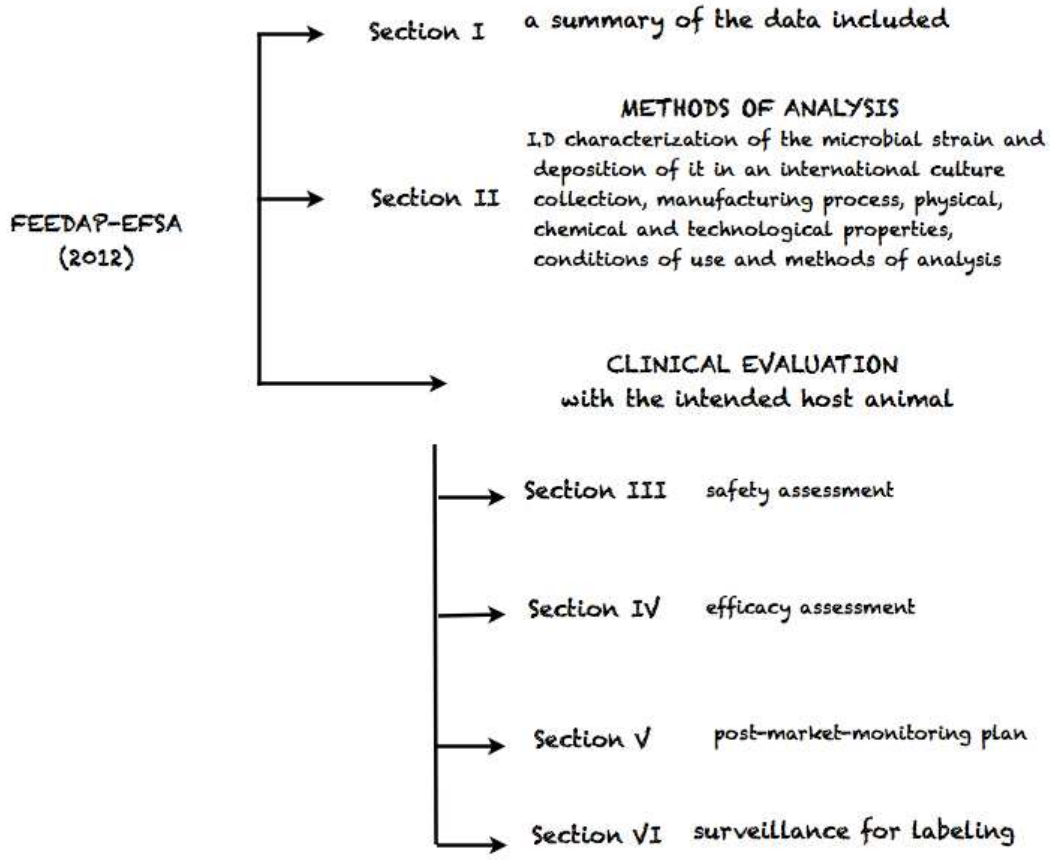

Figure 10. EFSAand FEEDAP recommendated evaluation process adopted from Guideline for evaluation progress for zootechnical additives of 2012

The recommended scheme regarding the evaluation of the probiotic potential in microorganisms shall be the same with the first three phases that FAO/WHO evaluation scheme of 2001 and 2002 reccomendsand can be found in Chapter 2 Section 3, assessed at strain level (Morelli & Capurso, 2012), withthe fouth phase regarding the targeted host organism/animal, for porposes of minimizing the in vivo studies.Further analysis of each phase

or section is presented, regarding the administration ofcolony forming microorganisms as additives in each target animal species,below.

Section I: summary of the dossier requirements for establishing the list and the characteristics of studies and information on substances, micro-organisms and preparations to be submitted with dossiers under Article 7 of 1831/2003/EC. The dossier shall include detailed reports of all the studies performed, presented in accordance with the numbering system proposed in the guidelines. The dossier shall include references and copies of all published scientific data mentioned and the copies of any other relevant opinions which have already been produced by any recognised scientific body. Where these studies have already been evaluated by a European scientific body following the legislation in force in the Community, a reference to the result of the evaluation shall be sufficient. Data from studies that have been conducted and published previously or coming from peer review shall clearly refer to the same additive as the one subject to the application for authorisation.

Section II: identity, characterisation and conditions of use of the additive; methods of analysis. The Identification (I.D) of the additive the name of the additive, a proposal for classification as it is referd in article 6 Annex I of 1831/2003/EC, a qualitative and quantitative composition, the purity and its physical state, shall be included. The characterization includes a description of the origin and the history of its modification, if any, its nomenclature, as well as a confirmation for its genetic stability and/or a description for toxic and virulence factors and/or the possibility of antibiotic production and resistance (cross- resistance). Finally a deposition of it in an international culture collection is required. Manufacturing process are advised to be described in a form of a caltivation flowchart. Physical chemical and technological properties include the stability of the strain and possible interactions with feed substances for calculation of the shelf life. Conditions of use includes a proposed mode of use, information for worker safety if the strain is supposed to be added in water. Finally, methods of analysis and reference samples are required to be included (IFIF, 2013)

Section III: studies concerning the safety of the additives are divived in four sub-sections. concerning the safety of use of the additive for the target animals, toxicological, microbiological, metabolism and residue studies as well as in certain cases tolerance studies for the evaluation of toxicity, have to be performed. Studies concerning the safety of the additive use for consumer include toxicity tests, metabolic tests, residue studies for the eshtablishment of withdrawl periods and a consumer safety assessment for the setting of MRLs based on the average daily intake (ADI). In the case of companion animals or pets, studies are insteadfocused on the owner taking into account potential hazards for young children. Studies concerning the safety of the additive for users/workers include an exposure assessment and miligating mesures in which an MSDS data sheet is required. Studies concerning the safety of the additive for the environment include a phase I and/or a phase II assessment for the calculation of predicted environmental concentration (PEC) where the calculated and Predicted No Effect Concentration (PNEC) values for each compartment shall be compared in order to refine the environmental risk assessment (429/2008/EC).

Section IV: studies concerning the efficacy of the additives are divided in long term and short term in vivo target specific studies, depending on the claimed effect and/or the proposed mode of use. Those are in vivo long term studies if the claimed effect relays the production or performance of the animal, short term or long term studies if the claimed effect on walefare has to do with the stress levels or the mortality as well as extrapolation of short term studies for claims on environmental concenquences. In cases that the quality of animal products are not the claimed effect specific residue studies are reqired in which, if literature reference are not available, an in vivo comparison of no-dose and highest dose takes place for physiological and metabolic conciderations (429/2008/EC).

Section V: Post-Market Monitoring Plan includes the design of the monitoring plan shall be detailed on a case-by-case basis and identify who (e.g. applicant, users) will carry out the various tasks that the monitoring plan requires, who is responsible for ensuring that the monitoring plan is set into place and carried out appropriately, and that there is a route by which the competent control authorities. The Commission and the Authority will be informed of any observed adverse effects, without prejudice to the provisions on supervision laid down in Article 12 of 1831/ 2003/EC.

For Pollinators the assesment it is far more complicated and significantly different but it can follow the same structure and orientation as above with the extrapolation of the requirements from relevant doccuments. EFSA has authorized the panel*EFSA 4 bees* in the specific matter. According to the European framework on animal health and welfare in 2013 EFSA prepared an Guidance for assessing the potencial risks to honey bees, bumble bees and solitary bees from the use of pesticides which includes microbial pesticides, and it can be used as well for extrapolation of information for the Risk assessment of zootechnical additives, taking into account that bacteria can be derived from flowers during pollination (EFSA, 2013). As regards the Residue qualification in honey and wax the paragraph 6.3.2.2 of Annex III in 429/2008/EC can be used. Moreover, the White Paper in Support of the Proposed Risk Assessment Process for Beespublished by FIFRA scientific advisory panel in 2012 for the quantitative risk assessment for the use of pesticides can be referd to for futher information(FIFRA, 2012). Finaly in the US, the environmental protection agency (EPA) published a Guidance for Assessing Pesticide Risks to Bees in 2014(EPA, 2014) and the Guideance on exposure and effects testing for assessing risks to bees in 2016(EPA, 2016). Sections I, II, V and IV can be applied for additives already authorized for major species as defined by Article 1(1) of 429/2008/EC.

A further analysis of references for the implementation of probiotic microorganisms as feed additives on each animal species is presented belowin **Table 5.**

Table 5. References for the implementation of probiotic microorganisms as feed additives on each animal species

SECTIONS	FOOD-PRODUCING ANIMALS INCLUDING SAMONS AND TROUT	FIN-FISHES, MINOR SALMONOIDS AND OTHER SPIECES	CASES OF MICROORGANISMS AUTHORIZED IN FOOD	NON-FOOD PRODUCING ANIMALS
I. SUMMARY OF THE DATA IN THE DOSSIER	Article 7(3)(h) of 1831/2003/EC , EFSA Guidance 2012 and Annex II: Section I of 429/2008/EC	For physiologically similar sp. as EFSA 2008 paragraph 1 and same summary of data can be added as described in Column 2 of row I	Same summary of data can be added as described in Column 2 of row I	Same summary of data can be added as described in Column 2 of row I
II. METHODS OF ANALYSIS	429/2008/EC, EFSA Guidance 2012; *I.D*; 429/2008/EC *Characterization*; 429/2008/EC *Manufacturing Process*; 429/2008/EC and IFIF, 2013 *Physical, Chemical and Technological properties*; if used in water 429/2008/EC and IFIF, 2013 *Conditions of use*; if added in water see EFSA 2011a as well as 429/2008/EC *Methods of analysis and reference samples*;429/2008/EC and EURL Guidance and IFIF, 2013		For holder specific same methods of analysis as described in Column 2 of row II	For holder specific same methods of analysis as described in Column 2 of row II
			For non-holder specific additives see at chapters 6.2 and 8.2 of 429/2008/EC	For non-holder specific see 429/2008/EC. Information and data from already authorized feed additives account as well; EFSA, 2011in addition with *Conditions of use*; if added in water see technical guidance EFSA 2011a and 429/2008/EC
III. TARGET SPECIFIC SAFETY STUDIES FOR:	Not required for QPS strains; EFSA Guidance 2012.	Not required for QPS strains ; EFSA Guidance 2012.	Not required for QPS strains and for authorized or approved strains in the European Union; EFSA Guidance 2012 and of 429/2008/EC	
	429/2008/EC, EFSA Guidance 2012 and IFIF, 2013	Presentation of most available recent formal assessments including their data; Paragraph 8.3 of 429/2008/EC)	Presentation of most available recent formal assessments	Requirements for different functional groups can be applied

SECTIONS	FOOD-PRODUCING ANIMALS INCLUDING SAMONS AND TROUT	FIN-FISHES, MINOR SALMONOIDS AND OTHER SPIECES	CASES OF MICROORGANISMS AUTHORIZED IN FOOD	NON-FOOD PRODUCING ANIMALS
		Tolerance studies required in cases were there are no data available from major relevant sp., having the same metabolism and physiology	including their data; 429/2008/EC	
Target Animals	In cases if added in water see EFSA 2011;. Paragraph 2.3 and IFIF, 2013 p. 40	*In vitroresidue* and *metabolism studies* are required if minor sp. EFSA Guidance 2012, *tolerance studies*; 429/2008/EC except if found a 10-fold dose safety assessment in relevant major sp. and/or if found a 10-fold of the highest recommended dose in major sp. no additional studies required for non-physiologically similar sp. or exempt sp. by Guidance documents; EFSA, 2011	In cases of similar or lower than for feed concentrations as in foods; metabolic capacity studies required; 429/2008/EC	Information on how to perform and report *tolerance studies* seeEFSA 2011; Tolerance studies for 28 days required in cases were there are no 10-fold-dose safety studies on monogastric animals, ruminants and poultry available for comparison.
Consumers	*consumer safety assessment is not required* for authorized or approved microbial species in the European Union; 429/2008/EC. types of *Toxicity tests*; IFIF, 2013 and requirements 429/2008/EC; EFSA, 2012b.	*Consumer safety assessment;* 429/2008/EC and EFSA, 2008a for the setting of MRLs	*Tolerance and residue studies* required for QPS strains in cases of different pattern metabolites or possibility of excessive consumer exposure as well; 429/2008/EC and IFIF, 2013	-
Owner	-	-	-	Assessment of potential hazards emphasized on children
User/Worker	429/2008/EC, EFSA, 2012b with exception on microencapsulated microorganisms and on no-respiratory sensitizers with justification	In cases were there is a foreseen change in exposure or have not assessed for other major sp.; 429/2008/EC and EFSA 2008b	-	In cases were there are not addressed assessments and measures for other sp. 429/2008/EC

SECTIONS	FOOD-PRODUCING ANIMALS INCLUDING SAMONS AND TROUT	FIN-FISHES, MINOR SALMONOIDS AND OTHER SPIECES	CASES OF MICROORGANISMS AUTHORIZED IN FOOD	NON-FOOD PRODUCING ANIMALS
Environment	If non-QPS or microorganisms found in QPS registry but the is isolated from a non-gastrointestinal environment or is rarely found in the environment see 429/2008/EC and EFSA, 2008a	For physiologically comparable major sp.; 429/2008/EC and EFSA, 2008a	Same summary of data can be added as described in Column 2 of row 11	-
IV. EFFICACY STUDIES	Evidence for efficacy can be extrapolated from evidence of mode(s) of action if at least one of the characteristics settled out on Article 5(3) of 1831/2003/EC can be satisfied with short term studies or end-points. In vivo long term studies are required; 429/2008/EC for minimum duration studies for target animals provided in a tabular form; EFSA Guidance 2012. Details on how to perform and report those studies see EFSA 2011	Evidence from approved physiologically comparable major sp. and known mode of action account as evidence of efficacy; chapter 6.4 of 429/2008/EC and sec. IV of EFSA, 2008b otherwise Annex II; Sec. IV as Annex II: sub-sec. 4.4 and Annex IV; table 6 of 429/2008/EC. Details on how to perform and report those studies see EFSA 2011	Performance of *efficacy studies* in cases of different function of microorganisms than in foods see at 429/2008/EC	In cases of already authorized additives for food; EFSA, 2008b. In cases were there are no authorized studies on monogastric animals, ruminants and poultry available or have different mode of action/effect *In vivo* long term studies for at least 28 days required; 429/2008/EC. Details on how to perform and report those studies see EFSA 2011
V. POST-MARKET MONITORING PLAN	A dossier is requested with particular documents as requested by 1831/2003/EC articles 6(1) and 7(3) for authorization whilst the form can be found in 429/2008/EC complying with the Feed Hygiene Regulation requirements of 183/2005/EC. Further description can be found in IFIF, 2013			
VI. LABELING	Labeling and specific labeling requirements are set in Annex III of 1831/2003/EC			

Abbrevations; Sp.; species, Sec.;Section, Sub-sec: subsection, p.: page

EFSA 2008a; Technical Guidance for assessing the safety of feed additives for the environment of EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2008

EFSA, 2008b; Guidance for the preparation of dossiers for additives already authorised for use in food as prepared by the Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2008

EFSA 2011; technical guidance on tolerance and efficacy studies in target animalsof EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2011

EFSA Guidance 2012; Guidance for the preparation of dossiers for zootechnical additives of EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012

EFSA 2012; Guidance on the assessment of additives intended to be used in pets and other non food-producing animals, of EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), as updated in 2012

EFSA,2012b: Guidance on studies concerning the safety of use of the additive for users/workers of EFSA Panel on Additives and Products or Substances used in Animal Feed (FEEDAP), 2012

IFIF, 2013;Comparison of Regulatory Management of Authorized Ingredients, Approval Processes, and Risk-Assessment Procedures for Feed Ingredients On behalf of International Feed Industry Federation, 2013

EU/ CRL Guidance; Explanatoy notes to applicants on chapter 2.6 Methods of analysis and reference samples of Annex II of 429/2008/EC and EURL-FA Administrative Guidance for Applicants for the reference samples and fee payments, 2015

3.1.4 Mode of action

In animal nutrition, great efforts have been made to study the mode of action of probiotics, although hard experimental data is still rather limited. The problem with probiotics is the lack of homogenus evidence as to their mechanism of action and of the effects on host animals is depended on the animal species and age, dose, time and even the mode of administration (IRTA, 2015). In addition, having in mind that the use of selectedmicroorganisms from different originscan exhibit similar beneficial effects,together with their variable survivability throughout the gut transist,it seems logical that their modes of action is also variable and will probably be based on more than one principles (Bernardeau & Vernoux 2013).

In correlation with the mechanisms of action reported at sub-section 2.1.4 of Chapter 2, the mechanisms may differ from one probiotic strain to another even for the same effect, thus being a difficult and complex area yet to be unravelled. In contrast with the proposal of the possible mechanisms of action on the control of intestinal pathogens for humans,some scientists (article 5(3)of 1831/2003/EC,Bernardeau & Vernoux 2013) have proposed that probiotic microorganisms regarding animals act beneficially with several mechanisms which can be summarized as two main modes of action or effects:

1. Interaction with hosts gastrointestignal epithelium and/or microflora
2. Interaction with hosts immune system

Interaction with hosts gastrointestignal epithelium and/or microflora

Probiotics are effective in certain cases, notably in newborn animals or those that have been treated with antibiotics, where they have the same effect as competitive exclusion products(Yirga, 2015). They are believed to improve the overall health of animals by improving the microbial balance in their intestingns.However, the modification of the intestignal microbial population seems to be the prime mode of action considering their broad beneficial effects. The interaction between the intestinal microbiota and the probiotic strains may be based on their aggregation with pathogenic bacteria, their capability to produce andimicrobial substances against the former including other specific substances, competition for adhesion to epithelial receptors or competition for nutrients.

Probiotics may also be useful in helping to boost weight gain and feed conversion rates. Most of the cases being used is as an alternative to antibiotic growth promoters.According to the National Office of Animal Health in a conference of 2013 (Sumner, 2014), antibiotic growth promoters are used to "*help growing animals digest their food more efficiently, get maximum*

benefit from it and allow them to develop into strong and healthy individuals". The amounts of antibiotics used have exerted a very strong selection pressure towards resistance among bacteria, which have adapted to this situation, mainly by a horizontal and promiscuous flow of resistance genes (SCAN, 2003;Balcazar *et al.,* 2006). Whilist their mode of action is unclear, it is believed that antibiotics suppress sensitive populations of bacteria in the intestines.

Probiotics are characterized by general positive mechanism of action in the host's system, with species specific level effects and rarely strain specific effects.

Competitive exclusion is the ability of normal microflora to protect the hosts from the harmful establishment of pathogens. Detrimental bacteria need to become attached to the gut wall to exert their harmful effects (Mc Donald *et al.,*2010). Probiotics may colonise and multiply in the gut, thereby blocking receptor sites and preventing the attachment of other bacteria including harmful species such as enteropathogenic *E. coli* or *Salmonella*. The mechanism of colonization is suggested to be associated with certain species within the microflora which can influence the expression of some messenger RNAs and of glycolypid conjugates on epithelial cells that may serve as receptors for the adhesion of bacteria. The concept of competitive exclusion indicates that cultures of selected, beneficial microorganisms, supplemented to the feed, compete with potentially harmful bacteria in terms of adhesion sites and organic substrates (mainly carbon and energy sources) (IRTA, 2015).

Undoubtedly, probiotics have the potential to decrease the risk of infections and intestinal disorders. As shown in in vitro studies by Hillman *et al.* (1995), growth of enterotoxic *E. coli* was successfully inhibited by different strains of *Lactobacilli* (Yirga, 2015). As reported by Berchieri *et al.*(2006), a combination of different lactic acid bacteria significantly reduced the levels of *Salmonella enteritidis* in fecal contents of broilers which had been orally inoculated with the pathogen. In piglets, attachment of enterotoxic *E. coli* to the small intestinal epithelium was inhibited by dietary supplementation with *Enterococcus faecium* (Jin *et al*., 2000;Steiner,2009;Yirga, 2015)

Furthermore, probiotic microorganisms, once established in the gut, may produce substances with bactericidal or bacteriostatic properties (bacteriocins) such as lactoferrin, lysozyme, hydrogen peroxide as well as several organic acids. These substances have a detrimental impact on harmful bacteria, which is primarily due to a lowering of the gut pH (Kelly and King, 2001; Conway, 1996). A decrease in pH may partially offset the low secretion of hydrochloric acid in the stomach of weanling piglets. These compounds may reduce not only the number of viable pathogenic organisms but may also affect bacterial metabolism and toxin production. Bacteriocins produced by lactic acid bacteria have been reported to be able to permeate the outer membrane of gram-negative bacteria and subsequently induce the inactivation of gram-negative bacteria in conjunction with other enhancing anti-microbial environmental factors such as low temperatures, organic acids and detergents (Alakomi HL, Saarela M & Helander IM., 2003; Yirga, 2015).

In addition, they can prevent amine synthesis. Coliform bacteria decarboxylate amino acids to produce amines, which irritate the gut, are toxic and concurrent with the incidence of diarrhea. If desirable bacteria prevent the coliforms proliferating, then amine production will also be prevented (Mc Donald *et al.,*2010).

Moreover, competition for energy and nutrients between probiotic and other bacteria may result in a suppression of pathogenic species (Ewing & Cole, 1994). The impact of probiotics, applied though feed or feed and drinking water, in comparison to antibiotic growth promoter (AGP, Avilamycin) on gut microflora of broilers has been demonstrated by Mountzouris *et al*. (2006). In total 400 day old broilers were fed corn-soybean meal-based diets with or without supplementation of either a newly-developed multi-strain probiotic feed additive based on *Lactobacilli, Bifidobacteria, Enterococcus* and *Pediococcus* (Yu *et al.,* 2008) or a commercial AGPs. Compared to the control and AGP treatment, the probiotic additive significantly increased the numbers of beneficial *Bifidobacteria, Lactobacilli* and Gram-positive cocci. Moreover, growth performance in birds fed supplemental synbiotics was similar as compared to birds fed the AGP (Steiner,2009)

Interaction with hosts immune system

Probiotic compete with pathogenic bacteria in adherence to the mucus layer covering the intestinal epithelium, interfering with the pathogen colonisation in the gut and, in most cases, modulating the host immune response. Intestinal morphology can be affected by dietary supplementation of different immunomodulatory substances / agents. The cellular components of innate immune system (macrophages and heterophils) protect the host from enteric infection. When intestinal microorganisms breach the intestinal epithelial barrier, these immune cells are recruited to the site of infection, where they kill the invaders using a variety of strategies, such as phagocytosis and oxidative burst. After toll-like receptor (TLR) activation, one possible outcome is the synthesis and release of proinflammatory cytokines. The presence of these cytokines modulates adaptive immunity. The manipulation of gut microbiome through administration of immunomodulatory agents can influence cell- and antibody-mediated immune response. The main target for immunomodulatory feed additives is the reduction of local inflammation and limitation of further impairment of immune function (IRTA, Yirga 2015).

As so the evaluation parameters for their effects are considered the end-poinds of each selected biomarker; the health status (performance), systemic immune responces like proinlafatory cytocines and immunoglobulins and local immune responces like intestignal microbiota composition, paremeters including the morphology of the gut structure and other zootechnical parameters (IRTA, 2015).

In order to describe the benefits of the application of substances or agents on the immune system, the end-points assessed in scientific articles can be classified intothree main groups for the majority of animal species: local immune response, systemic immune response,

and health status. The most evaluated parameters were intestinal microbiota and gut structure (local immune response), immunoglobulins and cytokines (systemic immune response), and performance (health status) (IRTA, 2015).

In the case of fish, the end-points are measured by immunological parameters and health status. Moreover the parameters that are chosen to be evaluated mostly are lysozyme activity, leucocyte count, complement activity, immunoglobulin quantification, respiratory burst and phagocytic activity (immunological parameters studied), and performance (health status) (IRTA, 2015).This may aid the development of the immune system by stimulation of the antibodies production and increased phagocytic activity (Mc Donald *et al*.,2010). As the immune system is engaged following exposure to probiotic bacteria, any hostile bacteria are also noticed, following increased surveillance by leukocytes, and thus potential pathogens are eliminated (Hughes & Heritage, 2002; Yirga, 2015)

Some probiotic strains such as Lactobacillus have proven to be capable of stimulating the immune system. Fuller explained the immune system to be stimulated in two ways. They can either migrate through the gut wall as viable cells or multiply to a limited extent or antigens released by the dead organisms can be absorbed and stimulate the immune system directly. It is the product of this change which induces the immune response. And currently, it appears to be some relationship between the ability of a strain to translocate and the ability to be immunogenic (Fuller, 1992; Yirga, 2015)

The development and activation of the humoral and cellular gut-associated immune system is largely associated by the development of the gut microflora. According to Lan *et al*. (2005), microbial communities can support the animal's defence against invading pathogens by stimulating gastrointestinal immune response.Therefore, an expected effect of the addition of probiotics to the gastrointestinal tract is an increase in normal microflora colonization with inhibition of the adhesion of harmful pathogens on the intestinalepithelium (Cho, Zhao & Kim , 2011; Yirga, 2015) thereby blocking receptor sites and preventing the attachment of other bacteria including harmful species. .

The gut is such a rich source of nutrients that it may seem unlikely that microorganisms could not find sufficient food for growth. Probiotics possess a high fermentative activity and stimulate digestion. Lactobacilli are known to produce lactic acid and proteolytic enzymes which can enhance nutrient digestion in the gastrointestinal tract (Yu *et al*., 2008; Yirga, 2015). Different studies demonstrated that probiotics maximized crude protein and energy digestibility compared with those in non-probiotic treatments (Yu *et al*., 2008; Yirga, 2015). However, it should be noted be that the environment only has to be deficient in one essential nutrient in order to inhibit microbial growth. In addition, the ability to rapidly utilize an energy source may reduce the log phase of bacterial growth and make it impossible for the organism to resist the flushing effect exerted by peristalsis (Cho, Zhao & Kim , 2011; Yirga, 2015)

Lactobacilli ferment lactose to lactic acid, thereby reducing the pH to a level that harmful bacteria cannot tolerate. Hydrogen peroxide is also produced, which inhibits the

growth of Gram-negative bacteria. These substances have a detrimental impact on harmful bacteria, which is primarily due to a lowering of the gut pH. A decrease in pH may partially offset the low secretion of hydrochloric acid in the stomach of weanling piglets. Moreover, live yeasts ferment sugars derived from the degradation of starch, thus competing with the lactic-acid-producing bacteria, and thereby stabilize rumen pH and reduce the risk of acidosis. Improvement in early digestion and intake is brought about by alterations in the numbers and species of microorganisms in the rumen .

In addition to the above discussed, other postulated effects include beneficial interaction with bile salts, increased digestive enzyme production, more efficient absorption of nutrients, and greater vitamin production. Several mechanisms have been proposed to explain the effects of probiotics and it is likely that the positive results reported in the different animal studies are due to a combination of some, if not all, of these (Mc Donald *et al.*,2010).

In the European Community there held about 20 microbial feed additives. The list of QPS strains, first established in 2007 is to be reviewed annually (EFSA, 2007) and it can been found as 'European Union Register of Feed Additives pursuant to regulation 1831/2003' regarding veterinary nutrition.

Examples on their mode of action or efficancy effects are listed at **Table 6**. Most of the recent appointments highlighted the importance of allocation of sufficient dose of probiotic bacteria in order to perform the desired beneficial effects on the host's health.

Table 6: Main effects of probiotics in animal administration

Claimed effects	**Polygastric animals**	**Monogastric animals**	**Aquatic organisms**	**Pets**	**Pollinators**
Treatment of digestive disorders	Wisener *et al*, 2014				
Reduction and protection of pathogens	Pravarez, 2006, Wisener *et al*, 2014,	Nurmi and Rantala, 1973, Chateau *et al*., 1993, Stern *et al*., 2001, Dalloul and Lillehoj, 2005, Pravarez, 2006 IRTA, 2015	Sugita et.al., 2002, Pravarez, 2006, Balcazar *et al.*, 2006 IRTA, 2015	Hawrelak et.al., 2005, Grześkowiak *et al*., 2015	Pravarez, 2006, Mahesh Pattabhiramaia h *et al*., 2010
Stabilization of the ruminal pH	Chiquette *et al*., 2008				
Increased feed conversion efficiency and	Doreau and Jouany, 1998, Zhang *et al*, 2015	Agazzi *et al*., 2015	Refstie *et al*., 2005, IRTA, 2015		

Claimed effects	Polygastric animals	Monogastric animals	Aquatic organisms	Pets	Pollinators
digestibility					
Improved milk yield and composition	Sharah et.al.,2002; Kritas et.al. 2006, Ayad *et al*, 2013; Maragkoudakis *et al*, 2010				
Improve the immune system activity	Spaniol *et al*, 2015	Cetin et.al., 2005, Panda *et al.*,2007 IRTA, 2015	Rodriguez-Lanetty *et al*, 2006, Marshal-Jones et.al., 2006, Skugor *et al*, 2008, Magnadottir, 2010; Nayak *et al*, 2010; Akhter *et al*, 2015, Grześkowiak *et al*., 2015, IRTA, 2015	Evans and Lopez, 2004 IRTA, 2015	
Treatment of mastitis	Espeche *et al*, 2012				
bind mutagens	Apás *et al*, 2014				
increase of body weight, development and enhanced performance	Bohmer *et al*., 2006, Samli et.al.,2007, Zhang *et al*, 2015, Agazzi *et al*., 2015 IRTA, 2015	Planik and Skott, 1980, Bohmer *et al*., 2006, Samli et.al.,2007, Avella *et al*, 2010, Agazzi *et al*., 2015	Grześkowiak *et al*., 2015		Kaznowski *et al*. 2005, Kazimierczak-Baryczko and Szymas 2006, Pătruică *et al*., 2011; 2012; 2013
reduction of the risk diarrhea	IRTA, 2015	Kyriakis et.al.1999, Ogawa *et al*., 2001; Casey *et al*., 2007, Agazzi *et al*., 2015		IRTA, 2015	
reduce the concentration of toxic gases such as NH_3, N_2O, H_2O_2 and methane in the excreta	Martin *et al*, 2010, Alazzeh *et al*, 2012, Karakurt *et al*, 2012, Jeyanathan *et al*, 2014	Dhama *et al*., 2008	Venkateswara, 2007	IRTA, 2015	
Protection against coccidosis		Lee *et al*., 2007			
Improve Production, decrease Contaminations,		Haddadin *et al*., 1996; Kurtoglu *et al*., 2004, Van Immerseel *et al*.,			

Claimed effects	Polygastric animals	Monogastric animals	Aquatic organisms	Pets	Pollinators
body weight gain, cholesterol and TAG concentrations in the egg yolk of Chicken eggs		2006,			
prolonged lifespan, cell proliferation and apoptosis	IRTA, 2015	Alexopoulos *et al.*, 2004, Paulius et.al.,2006	Rodriguez-Lanetty *et al*, 2006, Skugor *et al*, 2008		Máchová *et al.* 1997, Kazimierczak-Baryczko and Szymas 2006, Pătruică *et al.*, 2011; 2012; 2013
Reduce oxidative stress status			IRTA, 2015	Grześkowiak *et al.*, 2015	
control of allergic disorders, anti-inflammatory effect	IRTA, 2015	IRTA, 2015		Grześkowiak *et al.*, 2015	
Obesity				Grześkowiak *et al.*, 2015	

3.1.5 Examples of probiotic function in different host environments

Under normal living conditions there would be no need for probiotics hence in the wild, the young animal rapidly acquires a protective flora from its mother and the environment. However, modern methods of breeding tend to limit the contact with the mother while provide certain foods in combination with an unnatural environmental condition, for example in the case of poultry, where after the egg is laid, the chick is permanently separated from their mother. The result is that the gut microflora is deficient in some of the normal components that are responsible for resistance to diseases (Ezema, 2013).

Polygastric Animals

Ruminants are herbivorous mamals with signs of a still ongoing evoloution. The most widely distributed group of mammals on earth, currently add up to about 150 domestic and wild species while economic interest lies mainly in the breeding of:

1. cattle
2. sheep
3. goats
4. water buffaloes

Ruminants are 'extreme selective' animals with high levels of digestive efficancy since they can digest indigestable plant cell walls -fibre- via fermentation from cellulolytic bacteria in their forestomachs and then digest it. Their stomachs is a phylogenetic peack of complexity with the rumen to have the most diverce microbiome (Hoffman, 1989) In that way they are able to assimilate nutrients from low quality plant-based feeds, through their digestive tract, which is uniquely designed and includes, in contrast to other mammals, a four-compartmentalized stomach consisting:

I. of the rumen (pregastric anaerobic fermendation mainly from bacteria of fibers and solid feeds)
II. the reticulum (rumen liquids and large feed particles, regurgitated subsequently for optimal digestion)
III. the omasum (liquids are filtered and various nutrients are being absorbed)
IV. the abomasum (enzymatic digestion of the feed)

From the physiological point of view, each chamber performs different processes. (Hofmann, 1989).

The vast microbial diversity is considered to be in the rumen for polygastric animals, were its microbiome is composed predominately of bacterial species but also of methanogenic archaea, flagellated and ciliated protozoa, fungi, and bacteriophages (Chaucheyras-Durand and Ossa, 2014)with populations at a level of 10^{10} (bacteria), 10^{8} (protozoa), 10^{7} (archaea) and 10^{3} (fungal spores) colony forming units per ml of rumen fluid (Deusch *et al.,* 2015) each contributing in hosts physiological parameters.

Many reasherchers have chalenched the modulation of the rumen microbiome. The optimized ruminal fermentation is essential in supporting health and productivity in the ruminants, by managing the presence and the abundance of various microbial members (Hofmann, 1989; Jami *et al*., 2014). Moreover the ruminal microbiome can be correlated with physiological and production parameters, such as milk composition, of farm animals.Studies compearing the microbiome with other biocompounds of different animals in the same species are held in order to find common potential candidate taxa for production animals. Toward this, one of the problems seems to be the cultivation of those in the laboratory media, hence less of 15% of the rumen bacteria can be cultured (Morgavi *et al.,* 2013).

The reduction of pathogens in animals was one of the most effective ways, according to EFSA, of reducing the contamination of foodstuffs and human poisonings (EFSA 2010)

The systematic use of antibiotics was gradually adopted as a common practice in animal husbandry, targeting, inter alia, the beneficial manipulation of ruminal metabolism via increasing the selection pressure on bacteria to become resistant causing normal genetic mutations(Van Boeckel *et al.,* 2015) or use as growth promoters (Landers *et al.,* 2012). Nevertheless, their rampant use as growth promoters in animal feed during the last decades raised gradually concerns, not only for the antibiotic residues in animal products and the emergence of drug-resistant microorganisms, rendering antibiotics ineffective but also for the well-being of the animals themselves (Landers *et al.,* 2012).

Antimicrobial-resistant bacteria (ARB's) have significant puplic health implications, causing important implications like non-effectiveness of medicine and spread of livestock diseases incidence. Moreover, other cosequences that potentially out-waight the long term effects of antimicrobials are water and soil pollution, loss of microbial biodiversity and decline of meat quality. However, numerous countries e.g. Brasil, Russia, India, South Africa, China, the US, Australia etc., still employ antibiotics in livestock production and it is foreseen an unprecedented increase in usage rate during the next decade (Van Boeckel *et al.,* 2015).

In recent years, manipulation of the ruminal microflora can be achieved for example by supplementation of microorganisms called probiotics and direct fed microbials (DFMs), are used widely in the livestock production, especially in the European Union where the use of antibiotics in this field has been completely prohibited (1831/2003/EC)

The vast majority of the applications concern cows and the pre-ruminant life of calves, whereas the number of respective studies for lambs, sheep and goats has increased over the last years. The application of probiotics and DFMsin ruminant productivity and health include:

1. treatment of digestive disorders and reduction of gut pathogens (Wisener *et al.,* 2014),
2. stabilization of the ruminal pH and prevention of rumen acidosis (Chiquette *et al.,* 2008),
3. enhanced animal performance, increased feed conversion efficiency and fiber digestibility (Zhang *et al.* 2015),
4. improved milk yield and composition (Maragkoudakis *et al.,* 2010;Ayad *et al.,* 2013),
5. stimulation of the immune system (Spaniol*et al.,* 2015),
6. treatment of mastitis(Espeche *et al.*, 2012, Suskovic *et al.,*2010) and
7. methane mitigation (Chiquette *et al*., 2008; Alazzeh *et al.,* 2012).
8. bind mutagens either present in feeds or formed due to stress or gastrointestinal infections has been recently also reported (Apás *et al.,*2014).

The probiotic preparations are delivered to ruminants mainly orally, directly or in the feed, or via vaccine directly in the rumen.However, the oral administration may compromise the probiotic efficacy due to the adverse conditions prevailing in the GIT. For ensuring the stability and viability of probiotics, the microencapsulationtechnology has recently come into use, providing protection and controlled deliverance of the probiotic preparation in the GIT (Qi *et al.,* 2011).

An overview of the respective literature reveals the broad applicability of the well-studied lactic acid producing bacteria (LAB) as probiotics and DFMs in ruminants. The use of *Lactobacillus, Enterococcus, Streptococcus* and *Bifidobacterium* species has been reported. Besides LAB, several other microorganisms have been studied for their probiotic potencial in ruminants. These include lactic acid utilizers, such as *Propionibacterium* and strains of *Megasphaera elsdenii*, as well as other bacteria, such as *Escherichia coli*, *Bacillus* andfibrolytic *Prevotella* species (Dhama *et al.,* 2008; Seo *et al.,* 2010; Rafat &Hussain, 2014; Puniya *et al.,* 2015). In two recent studies, the use of cellulolytic *Ruminococcus* species in buffaloes and reindeers resulted in the beneficial modulation of their rumen microbiome (Kumar and Sirohi, 2013; Præsteng *et al.,* 2013).

While the most bacterial probiotics are highly efficacious in pre-ruminant calves, probiotic yeast and fungi, such as *Saccharomyces cerevisiae* and *Aspergillus oryzae,* respectively, have shown greater benefits for adult ruminants (Callaway *et al.,* 2014). It has been demonstrated that their use positively influences certain bacterial populations and the fermentation patterns in the rumen (Pinloche *et al.,* 2013).

Moreover, the use of propionibacteria in redusing methane production and total gas production in vivo has been performed in order to reduce Green House Gases (GHG). Bacteria, Fungi and protozoa are involved in CH_2 production mainly from CO_2 and H_2 releashed during fermendation prossess of feed. Butyrate and acetate have been linked biomarkers for the decrase of CH_4 emission (Nozière *et al.,* 2014). Probiotics have been studied aswell but they may not be a solution to this (Martin *et al.,* 2010, Buddle *et al.,* 2011Alazzeh *et al.,* 2012).

The interest for identifying candidate probiotics for ruminants is gradually focused on the autochthonous microorganisms from the various niches of the animal-target, for ensuing use in the tract from where they initially isolated (Nader-Macías *et al.,* 2008,Techera *et al.,* 2013). For example, comparison of the probiotic characteristics among isolates of dairy and animal rumen origin revealed that the latter were more tolerant in the presence of bile salts and exhibited higher inhibition against pathogens (Jose *et al.,* 2015). These findings show that the adaptation of the microorganisms in a specific ecosystem could play a significant role in the selection of probiotic candidates and that the probiotic efficacy of selected isolates on the host might be origin dependent. Furthermore, the use of rumen inhabitants as probiotics will result in enhancing the existing beneficial gut microflora, which seems to be a more mild method of gut microbiome manipulation than introducing ecosystem-irrelevant microbes (Kumar and Sirohi, 2013). Therefore, the niches of the ruminant GIT constitutes a rich and diverse reservoir for mining potentially novel probiotics (Tellez *et al.,* 2015).The boost in the development of high-throughput sequencing techniques revealed the abundance of uncultivable bacteria in the rumen ecosystem in comparison to the data obtained using only conventional microbiological methods (Cho *et al.,* 2011). The recent accumulation of metagenomics studies on the rumen microbiome can provide a vast body of information concerning not only the composition and the function of the respective microflora but also its interaction with the host animal and the feeds (Morgavi *et al.,* 2013).

In monogastric animals, strains of *Lactobacilli, Bacillus subtilis* and *Streptococci* have been used as probiotics. In ruminant animals, the application of yeast (*Saccharomyces*

cerevisiae) in the form of live culture, or dead cells with culture extracts, has proved successful in beneficially modifying rumen fermentation.

Yeast cultures can stimulate forage intake by increasing the rate of digestion of fibre in the rumen in the first 24 hours after its consumption. Overall digestibility is not affected. It is likely that this improvement in early digestion and intake is brought about by alterations in the numbers and species of microorganisms in the rumen. The precise means by which the effect is achieved have not yet been con- firmed, but there are a number of probable mechanisms .

It is thought that metabolites of dead and live yeast cells (B vitamins, branched- chain fatty acids, amino acids and peptides) stimulate the growth of the bacterial species Megasphaera elsdenii.This utilises the lactic acid produced from the rapid fermentation of starch and sugars associated with high-concentrate diets. Live yeasts ferment sugars derived from the degradation of starch, thus competing with the lactic-acid-producing bacteria, and thereby stabilise rumen pH and reduce the risk of acidosis. Live yeast cultures also scavenge oxygen in the rumen, helping to maintain anaerobic conditions and favouring the growth of cellulolytic bacteria. The increase in forage intake can result in improved liveweight gain, milk yield and milk fat content, although the effects are often small in dairy cows. The addition of yeast to intensive beef diets has increased daily liveweight gain and food conversion efficiency. Improved fibre digestion has also been reported in horses when yeast cultures have been given (McDonald *et al.,* 2010).

There are hundreds of bacterial strains that inhabit both animal and human gastrointestinal tracts. These bacteria include harmful or toxic bacteria that colonize within the digestive tract and produce toxic waste products which lead to gas or bloating, diarrhea, constipation, ulcers or more serious events like food poisoning, and beneficial bacteria. Thus, offering the possibility to exert a positive and completely natural effect on health, well-being and performance of the animal through its autochtonous microflora (FEFANA, 2005). Yet, the beneficial microorganisms produce enzymes that complement the digestive ability of the host, and their presence provides a barrier against invading pathogens (McDonald *et al.,* 2010).Probiotic administration to animals seems to promote livestock production and health in general. Multi-strain supplement probiotics may be more effective in comparison with single strain administration due to their synergistic effect on colonization, different type of action according to the type of spieces and a range of antimicrobial effect (Collado *et al.,* 2007, Timmerman *et al.,* 2004).

The types of probiotics administrated to animals are mainly bacteria, yeasts and fungi. Probiotics have been reported to enhance the growth of many domestic animals including cows (Doreau & Jouany, 1998) and being effective in chickens, pigs and pre-ruminant calves; whereas yeasts and fungal probiotics such as (*Saccharomyces cerevisiae*) and Amaferm (*Aspergillus oryzae*) have given better results in adult ruminants (Fuller, 1999). Microorganisms used as probiotics include those derived from the *Lactobacillus, Streptococcus, Enterococcus, Bacillus, Clostridium, Bifidobacterium* genera and *E. coli* Nissle 1917 (Kruis *et al.,* 2004).

Beyond DFMs and probiotics, other methods have developed considering to have the same effect. For example the use of vaccines with bacteria and viruses containing plasmid DNA or cytocines or other co-stimulatory molecules and nanoparticle-mediated plasmid delivery has been tried and seems quite effective for the modulation of the immune system but secondary trials have to be performed (Dhama *et al.,* 2008). Furthermore, non-live products from fermentations of probiotic microorganisms have been efficiently employed in ruminants (Bernard, 2015). In a recent study, the authors demonstrated that there was no evident benefit from the supplementation of live LAB when compared to the administration of non-live probiotic extracts (Jenkins & Jenkins, 2014). Among the various studies performed, even kefirhas been examined as a probiotic supplement in ruminants, but its administration did not affect significantly the physiological parameters of the animals (Ataşoğlu *et al,* 2010).

Monogastric Animals

Monogastrics are classified as animals having one simple or single-chambered stomach with the main agricultural species being:

- pigs
- poultry
- horses

The gut microbiota of pigs mainly consists of bacteria while a small percentage of archaeal sequences dominated by the *Methanomicrobia* and *Thermococci* have been also identified (Lamendella *et al.,* 2011; Isaacson *et al.,* 2012).

In the poultry gastrointestinal track (GIT), 13 phyla of bacteria were discovered with *Firmicutes*, *Bacteroidetes*, and *Proteobacteria* being the more representative ones with up to 900 and 500 species in chicken and turkey gut, respectively. Of all the species found, only 117 out of 900 and 69 out of 500 are established genera of bacteria with the most predominant genera in both chicken and turkey being *Clostridium*, *Ruminococcus*, *Lactobacillus*, and *Bacteroides*. Besides bacteria, the poultry GIT is also inhabited by methanogenic archaea, fungi and viruses (Yeoman *et al.,* 2012, Pan & Yu, 2014).On the other hand, the horse GIT in full is inhabited by bacteria as well, but also archaea, fungi and protozoa are also present (Daly *et al.,* 2001).

The composition and activity of intestinal microflora has a crucial impact on the animal health, growth and performance as a whole. After the ban of antibiotics as animal growth promoters in the European Union, Korea and Japan, probiotics gained ground as they present a variety of beneficial effects including, among others, promotion of gut health and homeostasis (Hou *et al.,* 2015).

The most frequently used probiotics in monogastric animals are yeasts (*Saccharomycesboulardii* and *S. cerevisiae*) and bacteria (*Lactobacillus* spp., *Enterococcus* spp.,

*Pediococcus*spp., *Bacillus* spp.) targeting the caecum and the colon (Agazzi *et al.,* 2015). The most common benefits of probiotics in monogastic animals are the

- increase of body weight (Agazzi *et al.,* 2015)
- the reduction of the risk diarrhea (Agazzi *et al.,* 2015)
- the improvement of feed efficiency and diet digestibility (Agazzi *et al.,* 2015)
- reduce the concentration of ammonia (Dhama *et al*., 2008)

Furthermore, probiotics have been assigned to play a significant role in providing supportive care to piglets during their initial part of life, while probiotics like Streptococcus faecium and Bacillus subtilis can reduce the concentration of ammonia in the excreta of poultry (Dhama et al., 2008). There are many microorganisms to be considered as potential probiotics but only a limited number of microorganisms seems to satisfy the necessary criteria.

In order to identify and detect the GIT microbiota from the animal gut and feces, several techniques have been developed based on biochemical, microbiological, immunological and molecular biological features. Among them, the expansion of high-throughput sequencing techniques exposed the plethora of non-culturable bacteria enabling the comprehensive characterization of the intestinal microflora of poultry and other monogastic animals (Danzeisen *et al.,* 2011;Cho *et al*. 2011). A full understanding of the intestinal microbiota and the genomic functions of its members, i.e. microbiome, will lead to the development of targeted probiotic strains and novel or improved strategies for effective microbiota modulation (Chambers & Gong 2011,Pan& Yu 2014, Choi & Chang 2015, Umu *et al.,* 2015). Next-generation sequencing studies in broilers and pigs gut discovered the age-related bacterial diversity revealing the importance of gut modulation in order to improve the animal health (Cho *et al.* 2011; Mohd Shauf *et al.,*2015).Compared to the other monogastric animals, there is only a limited number of studies characterizing the equine gut microflora using culture-independent methods (Daly & Shirazi-Beechey 2003,Hastie*et al.,* 2008, Yamano *et al.* 2008,Shepherd *et al*. 2011). However, as these characterization techniques have been recently developed, the results are not always successful (Sachsenroder *et al.,* 2014).

Aquaculture

Aquaculture is the farming of aquatic organisms and it involves the cultivation of freshwater and saltwater populations under controlled conditions. Compared to commercial fishing, this activity allows a selective increase in the production of species used for human consumption, industry or sport fishing. Due to overfishing of wild populations, aquaculture has become an economic activity of great importance around the world over the last decades

(FAO, 2012). Aquaculture has become an important economic activity in many countries. In large-scale production facilities, where aquatic animals are exposed to stressful conditions, problems related to diseases and deterioration of environmental conditions often occur and result in serious economic losses (Balcazar *et al.,* 2006). Importance of aquaculture product is set to increase dramatically as a result of overfishing of the world's waters and an increasing demand for seafood.A significant issue affecting production is the loss of stock through disease Diseases caused by *Vibrio spp.* and *Aeromonas spp.* are commonly implicated in episodes of mortality (Kesarcodi-watson *et al.,* 2008).

Prevention and control of diseases have led during recent decades to a substantial increase in the use of veterinary medicines (Balcazar *et al.,* 2006).

However, a growing number of scientific papers currently deal with probiotics and prebiotics in aquaculture in order to survey the state of the art and pass from their empirical use to their scientific approach.

Probiotics can be provided to the host or added to its aquatic environment in several ways: (i) addition via live food (Gomez-Gil *et al.,* 1998); (ii) bathing (Austin *et al.,* 1995; Gram *et al.,* 1999); (iii) addition to culture water (Moriarty, 1998; Spanggaard *et al.,* 2001); (iv) addition to artificial diet (Rengpipat *et al.,* 2000). For example, it has been reported that daily inoculations of larval white shrimp (*L. vannamei*) tanks with probiotic bacteria at a density of 10^5 cfu ml/1 prevented colonization by pathogenic bacteria during larval culture (Peeters &Rodrıguez, 1999; Balcazar *et al.,*2006).

The development of probiotics applicable to commercial use in aquaculture is a multistep and multidisciplinary process requiring both empirical and fundamental research, full-scale trials, and an economic assessment of its use. Defined procedural strategies have been proposed on the selection and evaluation of probiotic candidates for farmed aquatic animals (Marlowe *et al.* 2014).

Possible benefitial effects linked to the administering of probiotics have already been suggested as:

I. competitive exclusion of pathogenic bacteria (Garriques &Arevalo, 1995; Moriarty, 1997; Gomez-Gil *et al.,* 2000; Balcazar *et al.,*2003, 2004; Vine*et al.,*2004a);
II. source of nutrients and enzymatic contribution to digestion (Sakata, 1990; Prieur *et al*., 1990; Garriques & Arevalo, 1995);
III. direct uptake of dissolved organic material mediated by the bacteria (Garriques & Arevalo, 1995; Moriarty, 1997); and others are still being investigated as:
IV. enhancement of the immune response against pathogenic microorganisms (Andlid Juarez & Gustafsson, 1995;Scholz *et al.,* 1999; Rengpipat *et al*., 2000; Gullian & Rodrıguez, 2002; Irianto &Austin, 2002; Balcazar, 2003; Balcazar *et al.,* 2004);
V. antiviral effects (Kamei *et al.,* 1988; Girones, Jofre & Bosch, 1989; Direkbusarakom Ruangpan & Na-anan, 1998; Balcazar *et al.,* 2006)
VI. improve water quality (Dalmine *et al.,*2011; Balcazar *et al.,* 2006)

A good pool of candidate probiotics is of major importance in the selection process, and for aquaculture it is vital to examine both autochthonous and allochthonous to the aquatic environment isolates (Gatesoupe, 2008). Whereas humans and terrestrial farm animals tend to have an intestinal microflora dominated by Gram-positive obligate or facultative anaerobes that of aquatic animals consists mainly of Gram-negative aerobic as well as obligate and facultative anaerobic bacteria (Vine *et al.,* 2006). Bacteria such as *Vibrio*, *Pseudomonas* and *Acinetobacter* constitute the predominant indigenous microbiota of a variety of marine fish species and crustaceans (Pandiyan *et al*, 2013) while, in contrast to saltwater species, the indigenous microbiota of freshwater animals is dominated by members of the genera *Aeromonas,Plesiomonas*, representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides, Fusobacterium, and Eubacterium* (Moriarty, 2003). Lactic acid bacteria are generally sub-dominant in aquatic organisms and represented essentially by the genus *Carnobacterium*(Balcazar *et al.,* 2006). Interestingly, despite the indigenous Gram-negative species, probiotics used in aquaculture belong mainly to the Gram-positive genera *Bacillus, Enterococcus, Lactobacillus* and *Carnobacterium* as well as to yeast specieswhen used as biological control or immuno-stimulatory agents. In contrast, probiotics used as antimicrobials in aquaculture belong essentially to the aforementioned Gram-negative genera (De *et al.,* 2014).

In the past, the information available on the intestinal microbiota of aquatic species was based on the use of conventional culture-dependent methods. Nowadays, applied molecular based approaches are used successfully for the analysis of bacterial community (Cruz *et al.,* 2012):

- 16S rDNA clone libraries (Han *et al.,* 2010; Iehata *et al.,* 2015);
- fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE) (Mc Intosh *et al.,* 2008; Sun *et al.,* 2012) and temporal temperature gradient electrophoresis (TTGE) (Navarrete *et al.*, 2010)
- fluorescent in situ hybridization (FISH) (Payne *et al.,* 2007).
- Also, in a limited number of recent studies, next-generation sequencing (NGS) approaches have been used and reveal a far greater level of species diversity in the gut microbiota of animals than previous studies that lacked an NGS approach(Merrifield and Ringo, 2014).

The use of gnotobiotic systems (animals cultured in axenic conditions or with a known reconstituted microbiota) can be an excellent tool to extend understanding of the mechanisms involved in host-microbe interactions of cultured animals (Dimitroglou *et al.,* 2011). This approach in parallel to the use of mutant strains, such as non-motile *Pseudomonas* mutants (Rawls *et al,* 2007) or yeast mutants (Soltanian *et al,* 2007) led in the past to the clarification of

genes involved in specific probiotic mechanisms in fishes and crustaceans, respectively. Further understanding of the mechanisms might also result from the use of tissue- or cell specific mutants expressing green fluorescent protein (GFP) or GFP variants as a powerful method for *in situ* monitoring of the presence and behavior of microbes that are intentionally introduced into the host organisms (Avella *et al.,* 2010; 2007). According to Tinh *et al.*(2008), GFP translational fusions of genes of interest in probiotics, when introduced into translucent larvae, might provide additional data on gene functioning.

The ability of probiotics to affect the ontogenetic development of animals by interfering with their gonad differentiation and maturation or progression to puberty and aging gains interest for future studies (Avella *et al.,* 2010). Indeed, microarray analysis was used in the past to evaluate alterations on the expression of genes involved in immune response, protein folding, cytoskeletal/structural proteins, vital cellular processes such as lipid metabolism, cell proliferation and apoptosis in aquatic organisms (Rodriguez-Lanetty *et al.,* 2006;).(Skugor *et al.,* 2008)

The genomic information that is generated from sequencing known probiotic bacteria provides clear understanding on the inherent probiotic properties (Ventura *et al.,* 2012). In aquaculture, the concept of probiogenomics is not yet widely recognized or even applied; however, recently the relevance of this perspective in aquaculture has been raised (Lazado & Caipang, 2014,Marlowe *et al.,* 2014)

Features correlated to certain modes of probiotic action in the aquatic environment are under investigation the last decades (Kesarcodi-watson *et al.,* 2008). Enhancement of colonization resistance and direct inhibitory activity against pathogens are considered important factors when probiotics are used for the prevention of bacterial diseases (Balcazar *et al.,* 2006). Potential probiotics can also be correlated to the growth promotion of cultivated fishes by producing a variety of extracellular enzymes (i.e. proteases, lipases, carbohydrases, phosphatases, esterases, lipases and peptidases) that facilitate the efficient absorption of nutrients (Bairagi *et al.,* 2002; Giri *et al.,* 2013).

For instance, the use of plant protein sources in the diets (Gatlin *et al.,* 2007) led to the investigation of the metabolic capabilities of probiotics, such as degradation of anti-nutritional factors, feature interrelated in the past with the improvement of the nutritional value of the feed of aquatic animals (Refstie *et al.,* 2005). Strains that enhance the decomposition of undesirable organic substances and improve the ecological environment by minimizing toxic gases such as NH_3, N_2O, H_2O_2 and methane have been proposed for potential use for the improvement of water quality (Venkateswara, 2007). Immunomodulation by probiotics has also gained great attention and assessment of phagocytic, respiratory burst, lysozyme, high serum peroxidase and complement activities, as well as modulation of cytokines production have been referred as potential strategies in order to find novel probiotic strains for aquaculture (Nayak *et al.,* 2010; Magnadottir, 2010; Akhter *et al.,* 2015).

Taking into consideration that probiotics for aquaculture are marketed in two forms, dry and liquid (Ghosh, Sinha & Sahu, 2008), an appropriate route of delivery of the probiotic to the host should be proposed. So far, literature refers to several ways that probiotics can be provided to the host or added to its aquatic environment, such as addition via live food, bathing, and addition to culture water and to artificial diet (Balcazar *et al.,* 2006). Bioencapsulation of probiotics has also been demonstrated to be a more effective way to introduce probiotics in the animal gut; in the case of some allochthonous bacteria this may be the only efficient route (Merrifield & Ringo, 2014).

The current literature is heavily focused on the bacterial microbiota and considerably less information is available on indigenous yeast, bacteriophages, archaea, microalgae and protozoans in aquaculture. Although it is debatable whether or not bacteriophages constitute *bona fide* probiotics, their influence on indigenous and probiotic bacteria must be taken into account for future studies especially after the 'kill the winner' hypothesis about their important role in shaping the mammalian gut microbiota (Mills, 2013). Moreover, bacteriophage therapy has been suggested in the past as an alternative for the prevention and treatment of microbial diseases in aquaculture (Nakai and Park, 2002). Even if many recent studies indicate their promising application (Castilho & Pereira, 2012), caution must be taken for their use in the future(Rao & Lalitha 2015).

Another recent concept in regards to the manipulation of gut microbiota of animals in aquaculture are synbiotics. The use of synbiotics is an important approach in order to explore in what way prebiotic administration may seed and maintain probiotic strains as the dominant species in the fish GI tract (Rurangwa *et al,* 2009). Despite recent progress in the field of synbiotics administration in aquaculture, there is limited information available on different aspects of synbiotics effects on fish species (Llewellyn *et al.,* 2014). To our knowledge, few studies so far have investigated the effect of synbiotics only in fish species since the first one in 2009 (Cerezuela *et al.*, 2011). In them, probiotics evaluated correspond to three bacterial genera, namely *Enterococcus*, *Bacillus* and *Pediococcus*, as well as prebiotics to FOS and MOS. The studied fish species have been rainbow trout (Rodriguez-Estrada *et al.,* 2009; Mehrabi *et al.,* 2012; Grze *et al.*, 2015), Japanese flounder (Ye *et al.,* 2011), yellow croaker (Ai *et al.,* 2011), cobia (Geng *et al.,* 2011), sea bream (Cerezuela *et al.,* 2013) and Atlantic salmon (Abid *et al.,* 2013) indicating better growth, feed efficiency ratio, improved immune responses and disease resistance of aquatic animals after synbiotic supplementation.

Pets

Dogs and cats are carnivores with a history of high protein diets. Today, cats and most dogs are on high carbohydrate diets living in urban areas and thus face similar life-style challenges with humans. The health and well-being of companion animals, just as their owners, depends on the gut microbes.However, as microbiota differences may facilitate exposure to pathogens and harmful environmental influences, it is prudent to search for novel

tools to protect dogs and cats and at the same time the human owners from pathogens.

Domestic dogs and cats live in conjuction with humans while benefiting from each other. Mutual interest has evolved companion animals into being a stable part of human life and therefore, the health and well being of pets have increasingly raised interest during last decades.

Companion animals have high numbers of microorganisms in the gastrointestinal tract (GIT), which in fact exceed in quantity those living in human gut. Nonetheless, both cats and dogs have distinct bacterial species that differ between each other and also vary among different dog and cat breeds, various gut niches and geographical areas. Microbial diversity and concentration increase along the length of the GIT. The prevalent bacterial phyla in the colon and faeces of both dogs and cats are represented by *Firmicutes, Bacteroidetes, Proteobacteria* and *Fusobacteria* as well as *Eubacterium* in cats. The microbial differences between dogs and cats are manifested in the microbial groups and species levels (Grześkowiak *et al.,* 2015). Molecular fingerprinting has revealed that every individual pet has a unique and stable microbial ecosystem (Suchodolski, 2011). A recent metagenomics approach estimated that, besides bacteria, the feline GIT microbiota comprises 0.02% fungi, 0.09% archea and 0.09% viruses with 99% of them being bacteriophages. The most commonly observed archaeal phyla belonged to *Chrenarchaeota* and *Euryarchaeota*, with the most abundant families being *Desulfurococcaceae* (54.8% of sequences), *Methanobacteriaceae* (40.6%), *Methanosarcinaceae* (5%) and *Halobacteriaceae* (2.7%) (Tun *et al.,* 2012). According to Handl *et al.* (2011),among fungi, *Aspergillus* and *Saccharomyces* are the most abundant genera in feline GIT microbiota. As for other animals, any disturbances within the gut microbiota of the pets may lead to the development of a multitude of diseases and disorders, such as diarrhea, allergies, obesity and stress symptoms (Lee & Hase, 2014).

The majority of probiotic strains for humans and animals belong to lactic acid bacteria (LAB) and for humans bifidobacteria.There have been many attentions in assembling potential probiotic strains isolated from cats and dogs origin, however, most of the probiotics for companion animals are not originally derived from the canine or feline GIT microbiota .

Possible benefits of the probiotic use in pets include modulation of the immune system, help in stress maintenance, protection from zoonostic diseases like protozoa parasites and ascariasis to antimicrobial multiresistancy which can be transfered the owner aswell, protection from infections caused by enteropathogens, increased growth and development, dogs with IBD, control of allergic disorders and recently also obesity (Grześkowiak *et al.,* 2015).

So far, the common mode of administration of probiotics to pets is oral by adding them to the pets feed *(Hutchins et al.,* 2013; Bybee *et al.,* 2011; Biagi *et al.,* 2007). Regarding the genera, which are used as probiotics in companion animals, these include mainly *Bacillus* spp. , *Lactobacillus* spp., *Bifidobacterium* spp. (Biagi *et al*., 2013), *Enterococcus faecium* (Benyacoub *et al.,* 2003; Vahjen & Männer, 2003; Marciňáková *et al.,* 2006; Bybee *et al.,* 2011; González-

Ortiz *et al.,* 2013) and only recently scientists started using as probiotics *Weissella confusa* and *Streptococcus thermophiles.*

In order to enhance survival of probiotics during passage through the GIT of pets, encapsulation of bacteria has been used so that a larger number of viable bacteria can reach the intestine. Starch, alginate, carrageenan and chitosan are included among the hydrocolloids used to encapsulate or to obtain films and coatings (González-Forte *et al.,* 2014).

Regarding the combination of probiotics and prebiotics, Swanson *et al.*(2002)were the first to study the effect of synbiotics, namely administration of FOS and/or *Lactobacillus acidophilus*, on the gut microbial populations, end products and nutrient digestibilities in healthy adult dogs. It was shown that FOS enhanced indices of gut health by positively reshaping gut microbial ecology and fecal protein catabolites, whereas *Lactobacillus acidophilus* was more effective when fed in combination with FOS rather than fed alone. Later on, Ogué-Bon *et al.*(2010)showed that GOS supplementation can sustain the growth of *Bifidobacterium bifidum,* when used as a synbiotic combination, in canine fecal microbiota, while Biagi *et al.*(2013)reported that the combination of with GOS a strain of *Bifidobacterium pseudocatenulatum* had some positive effects on the intestinal microbiota in cats.

Bees

Ecosystem services, defined as the benefits to human welfare provided by organisms interacting in ecosystems, are considered to be at risk (Cane *et al.,* 2007) Pollination by wild animals is a key ecosystem service for the sexual reproduction of many crops and the majority of wild plants, providing calories and micronutrients to humans. As a pollinator, the honey bee, *Apis mellifera*, is a key species for agricultural production and contributes to the human food supply represending the most economically valuable pollinators of crop monocultures worldwide (Cane *et al.,* 2007). Recent losses of *A. mellifera* and bumble bees (genus *Bombus*), and the potential association of these declines with various infectious agents, call for a better understanding of the bees' microbiota (Genersch, 2010; Evans and Schwarz, 2011). Honey bees pool resources, divide labor and correspond in highly structured social colonies. Sterile female worker bees dominate within colonies, in which they initially clean cells, rear brood and store food, then they leave the hive and search for pollen and nectar (Seeley, 1985). With regard to social insects, group living can facilitate the transmission of not only parasites but also beneficial microbes. Adult honey bees and bumble bees have recently been shown to harbor a specialized and surprisingly species-poor community of bacteria in their gut. These specific bacteria appear to be absent in solitary bee species, suggesting that a stable association with their hosts may be facilitated by sociality in these groups of corbiculate bees (Koch & Schmid-hempel, 2011).

Many insects have co-envolved non-pathogenic with microoganisms that provide benefits to both partners. That nutritional symbiosis is found in multiple insect lineges that subsist on unusual or low-nutrient diets (e.g sap,blood, wood) (Martison *et al*., 2010).

Independent studies of bacterial community profiles based on 16S rRNA sequences show that workers of *A. mellifera* and some *Bombus* species consistently harbor an offbeat gut microbiota not shared with solitary bees(Koch & Schmid-hempel, 2011). The most common bacteria in solitary bee species are a widespread phylotype of *Burkholderia* and the pervasive insect associate, *Wolbachia*. In contrast, several social representatives of corbiculate bees do possess distinctive bacterial phylotypes. The microbiota of worker bees consists of eight distinct species or phylotypes, i.e. closely related strains with ≥97% sequence identity in 16S rRNA sequences, hereafter referred to as species. These include three Gram-positive species, namely two closely related *Firmicutes* within *Lactobacillus* and one within *Bifidobacterium*, and five Gram-negative species, namely one β-proteobacterium with the Candidatus name "*Snodgrassella alvi*," two closely related γ-proteobacteria, one with the Candidatus name *"Gilliamella apicola*" and two α-proteobacteria (Martinson, Moy and Moran, 2012).

The application of probiotics in bees is achieved through feeding, with *Lactobacillus* and *Bifidobacterium* being the main genera used until now. Máchová *et al.*(1997) were the first who added probiotics, without specifying the microorganisms used though, into sugar syrup in order to feed honey bees (*Apis mellifera*) and noticed that this improved bee survival. The next attempt was not until seven years later, and it was demonstrated that probiotics including *Bifidobacteriuminfantis*, *B. longum, B. breve, Lactobacillus rhamnosus, L. acidophilus, L. reuteri, L. casei and L. plantarum* enhance immune responses in bees by stimulating the production of antimicrobial peptides against *Paenibacillus* and *Ascospaeraaapis* infections (Evans & Lopez, 2004). Kaznowski *et al.*(2005) used *Lactobacillus* spp., *Pediococcus acidilactici*, *Bifidobacterium bifidum* and *Enterococcus faecium* as supplements to pollen substitute in feeding honey bees. It was shown that in order to achieve increase in dry mass and crude fat level it was sufficient to supply probiotics only in the beginning of the feeding period, directly after bee emergence. These results have been confirmed by Kazimierczak-Baryczko and Szymas (2006), who used the same species and who also showed that the addition of probiotics in pollen substitute, prolonged bee lifespan and stimulated the growth of the faucial gland and fat body. Moreover, administration of *Lactobacillus* spp*., Bifidobacterium* spp*., Saccharomyces boulardii* and *Streptococcus thermophilus* through sugar syrup resulted in better colony development, a longer life-span and enhanced development of wax production (Pătruică *et al.,* 2011; 2012; 2013). It seems, however, that for probiotics to be efficient they have to be tailored for bees (Johnson *et al.,* 2014).

In recent years, molecular methods offer great potential for the phylogenetic identification of probiotic microorganisms in bees (Mattila *et al.,* 2012, Tajabadi *et al.,* 2013). Oloffson andVásquez (2008) detected and identified novel lactic acid bacteria, mainly lactobacilli, as well as bifidobacteria in the honey stomach of honey bees mainly belong to the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* by employing 16S rRNA sequencing

since it's a 'gold standard' for identification and phylogenetic analysis of LAB (Tajabadi *et al.,* 2013). Using the same method, Tajabadi *et al*. (2013) detected Lactobacillus spp. that L. plantarum, L. pentosus, and L. fermentum were the dominant lactobacilli in *Apis dorsata* honey comb, which could be explored as a source of new bacteria with probiotic potential in honey bees. Moreover, in prevews work the predominant LAB in the honey stomach deeper understanding of the complex host-microbial interactions might also result from the use of tissue- or cell specific mutants expressing green fluorescent protein (GFP) or GFP variants. In this direction, Hyršl *et al*. (2015) have successfully used a mutant of *Photorabdum luminscens* that expressed GFP in order to track the nematobacterial infection in bees.

3.1.6 Legislation on animal probiotics

The approval of probiotics for use in animals follows essentially the same approach as that for humans, which is largely dependant on the efficacy and toxicity of the strains.

Europe

The European Community food law, enshrined in Article 11 of Regulation178/2002/ECof the European Parliament and of EU Council, that food and feed produced or imported for placing on the market within the Community must comply with the relevant requirements of Community legislation or with conditions recognized by the Community to be at least equivalent thereto while subjecting additives for use in animal nutrition to requirements equivalent to those applying to considering feeding stuffs equivalent with foods. Live microorganisms, together with enzymes and feed additives of biological origin were added to the list of feed additives regulated by the European Union in the 1980s due to the emerging market trends. The term "probiotics" have been rejected on the grounds of being too generic. In 2002, under the framework of establishing the European Food Safety Authority(EFSA), a new draft regulation would group microorganisms as "zootechnical additives," defined as agents producing beneficial effect on gut microflora. This proposal was adopted in 2003, when the European Commission passed a new regulation 1831/2003/EC of the European Parliament and of the Council on additives for use in animal nutrition. The classification of additives according to the regulation mentioned, is based on the functionality of the microorganism administrated. Furthermore considering probiotics, can be defined and classified in five categories; Technological additives, sensory additives, nutritional additives, zootechnical additives and coccidiostats/himstomonostats.

The scientific Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) of the European Food Safety Authority assesses the safety and efficacy of feed additives under the Regulation No. 1831/2003/EC, while the Regulation No.429/2008/EC includes general provisions for proving the efficacy of feed additives. Efficacy studies should be designed to demonstrate the effect(s) of the additive by targeting sensitive parameters in comparison to a negative and, optionally, a positive control group. The FEEDAP Panel has issued a series of guidance documents to help the applicants in the preparation of dossiers. In these guidance documents, the provisions for demonstration of efficacy required by Regulation No 429/2008/ECare described in more detail. No specific guidance from the FEEDAP Panel exists for the assessment of this type of feed additives.

Moreover, when considering organic aquaculture animals, according with the Regulation No710/2009/EC,when a health problem arises, a vettenary treatment with authorized probiotics can be used.

Authorization of feed additives is granted by The European Food Safety Authority (EFSA), which evaluates the data submitted on efficacy, safety, and toxicology of the feed additive. Once the Commission is satisfied with the data, it prepares a draft regulation to grant authorization, following the procedure involving Member States within the Standing Committee on the Food Chain and Animal Health—Animal Nutrition. Authorizations are granted for specific animal species, specific conditions of use and for 10-year periods. Although the registration and approval can be interpreted as fairly complex, it can be argued that this is critical to ensure safety of probiotics used as feed additives that ultimately contributes to their efficacy.

A list of permitted claims on the basis of the data must be presented and submitted, containing an established cause and effect relationship between the consumption of the strain and the claimed effect in order to comply with the requirements. The rules for applications are settled at the907/2013/EC.The authorizations of health claims have to be submitted to the national competent authority of a Member State. Then, if the application is valid, it goes forward to the Authority. At last it is have to be authorized by the Commission.

After long-term studies, there held several arguments that confirm through a series of studies that probiotics play an important role in enhancing and promoting animal health.

Considering the intention of organizations and the EU to end all use of antibiotics as growth promoters, the need for additional strategies to modulate the gastrointestinal environment and microflora metabolism became a priority, where the use of pre- and probiotics offer an alternative solution.

The FEFAC position concerning medicated feed in general and the EU legislation (COM (2014) 556 2014) supporting the “One health” strategy aiming at reducing the use of antibiotics in livestock and companion animals along the principle “as much as needed, as little as possible”. Feed additives in general can not be put on the market unless authorisation has

been given following a scientific evaluation demonstrating that the additive has no harmful effects, on human and animal health and on the environment.

Other Countries

As regards the United States of America the FDA uses other terms for live microbes for regulatory purposes; live microbes used in animal feeds are called “direct-fed microbial”. Regarded as the authority and reference on feed additive policy, the Association of American Feed Control Authority (AAFCO) published a list of microorganisms approved as direct-fed microbial products and the United States Environmental Protection Agency (US EPA)especially as regards bees. For Japan the Food and Agricultural Materials Inspection Center (FAMIC) takes place for the evaluation of zootechnical additives.(Lee & Salaminien, 2009)

2.1.7 Where the research is going beyond the feed probiotic framework

Regarding ruminants

The concept of using bacteriophages for manipulating certain microbial populations in ruminants has been also studied (Callaway *et al.,* 2008). Although phages present high host specificity, their efficient application requires the identification of the bacterial target in the rumen. To prevent bacterial resistance the use of phage cocktails is recommended. In a recent study, a cocktail of designed bacteriophages was successfully employed as a biocontrol means against the gut pathogen *E. coli* in rat model animals and the results were promising for possible future use in ruminants (Abdulamir *et al.,* 2014)**.** An effective treatment demands the monitoring of the developing resistance mechanisms, the use of newly isolated phages from the rumen environment and even the development of new phages in the laboratories. Furthermore, the use of isolated lysins instead of whole bacteriophages could be a promising alternative. However, there are only few data available about the rumen virome. Recent studies on the rumen bacteriophages and their interactions with the rumen bacteria constitute an initial attempt to study the rumen virome in depth, helping to obtain new insights probably exploitable in the manipulation of rumen microbiome (Ross *et al.,* 2013). The detailed characterization of the rumen virome would be of great significance, since the endemic ruminal phages could prove to be either a useful probiotic tool (Hallewell *et al.,* 2014)or a drawback for the probiotic interventions in the animals (Kropinski *et al.,* 2012). Additionally, regarding the potential risk associated with the probiotic use of phages in lactating ruminants and the possible contamination of milk and dairy products further research is needed. If the adverse effect in dairy manufacturing is demonstrated, their application could be limited to meat producing animals.

The application of probiotics or the so-called DFMs could also play a decisive role in the mitigation of rumen methanogenesis, since the reduction of the enteric methane emissions could be attained through the enhancement of rumen fermentation efficiency and the augmentation of animals' productivity (Karakurt,Aydin & Aydiner, 2012). The environmental impact of the ruminant-derived methane is of considerable importance for the sustainability of livestock, since it is accountable for 25% of the global methane emissions produced by anthropogenic activities (Buddle *et al.,* 2011). The use of probiotic acetogenic bacteria and yeasts for decreasing methane emissions has been studied but further research is needed since the respective results are inconclusive. Another interesting aspect is the use of probiotics for controlling specifically the protozoal population in the rumen, since it has been reported that methanogens found both attached and inside ciliate protozoal cells are responsible for 9-37% of the enteric methane production (Martin *et al.,* 2010). According to Nozière *et al.* (2014)The proportional correlation among rumen protozoa and methane emission has been confirmed using a meta-analysis approach. Recently, the availability of genome projects on rumen methanogens can provide information about the dominant microorganisms implicated in methane production, e.g. methanogenic archaea (Leahy *et al,* 2013), leading to a more targeted selection of probiotics and DFMs.

The advance of rumen-protective technologies providing shielding from ruminal digestion, such as encapsulation, may become useful tools for the eventual use of selected probiotics in ruminant feeds (Callaway *et al.,* 2008). A wide variety of factors, such as the growth environment, the animal species and breed, the age and physiological state of the animal, the diet, the nature of the probiotic preparation used (e.g. type of microorganism, live culture or lyophilized cells) and even its dose, seem to affect the outcomes of probiotics' utilization in livestock.

The use of recombinant microorganisms with probiotic properties in ruminants have been also documented. The most successful study concerns the genetically modified bacterium *Butyrivibrio fibrisolvens*, in which a dehalogenase for fluoroacetate encoding gene from the soil bacterium *Moraxella* species was introduced (Gregg *et al,* 1994). The modified organism was able to degrade the toxic fluoroacetate present in forage plants. The results were encouraging since the microorganism survived in the rumen of sheep and cattle studied without the loss of the respective gene (Gregg *et al.,* 1998; Padmanabha *et al.,* 2004). The same species was also used for the creation of a recombinant xylanolytic strain. A plasmid containing a xylanase gene from *Neocallimastix patriciarum* was successfully inserted into *Butyrivibrio fibrisolvens*and although the modified microorganism had enhanced capacity for xylan degradation, it failed to persist in the rumen (Krause *et al.,* 2001). The recent information obtained from various sequencing projects and databases reveals the abundance of specialized microorganisms in the rumen. Thus it would be difficult for genetically engineered superbugs to fully colonize the ruminal microbial ecosystem and exert on the host the benefits for which they have been designed (Callaway *et al.,* 2014). Furthermore, the use of genetically modified microorganisms raises concerns about their impact on the host animal and the possible spread of the respective microorganisms in the surrounding environment and even the consumers. Hence their use in ruminant production should be carried out with the utmost caution.

Regarding monogastric animals

Although the native gut microflora is commonly used as a pool for probiotic candidates, the use of genetically modified strains as probiotics in monogastric animals is of ongoing interest (Sieo *et al*. 2005). A species commonly used for genetic engineering in poultry is *Lactobacillus reuteri*. A lot of research has been made using strains of this species expressing heterologous genes in a poultry diet with encouraging results on the growth performance and welfare of animals (Liu *et al.,* 2005, Liu *et al.,* 2007, Yu *et al.,* 2008, Li *et al.,* 2014). Since genetic engineering approaches have positive results in poultry, research is currently focusing on genetically modified strains capable of expressing more than one heterologous gene (Wang *et al.,* 2014). Apart from poultry, genetic engineering is also used in pigs either therapeutically, e.g. in pancreatic insufficiency, or as feed additives enhancing livestock production (Yin *et al*., 2010;Drouault *et al.,* 2002)

Bacteriophages (phages), virus infecting bacteria, have been proposed as valuable candidates as therapeutic agents, based on their capacity to invade and disrupt bacterial metabolism causing the bacteria lysis. Phages are host specific, preventing the destruction of most of the healthy flora, and they are able to multiply exponentially at the site of infection. Phages are harmless for animals, plants and for the environment (Sulakvelidze,Alavidze & Morris, 2001;Oliviera *et al*., 2010).

The idea of using bacteriophages to manage or eliminate zoonotic bacteria in poultry husbandry has been established as a cost-effective approach with significant advantages compared to antibiotics. Bacteriophage therapy has been shown to have efficacy to treat many diseases of plants, animals, and humans (Kutter & Sulakvelidze, 2005;Atterbury *et al*., 2007, Huff *et al.,* 2014). It is of interest that one of the first studies on the efficacy of bacteriophage to treat diseases was to prevent fowl typhus (d'Herelle, 1926). Bacteriophages can be used to both prevent and treat colibacillosis in poultry (Huff *et al.,* 2002a,b, 2003a,b, 2006, 2010, 2013) and in controlling necrotic enteritis (Miller *et al*., 2010; El-Gohary *et al.,* 2014)

The chicken gut microbial imbalance frequently caused by broad-spectrum antibiotics is avoided using host-specific bacteriophages. These bacteriophages are naturally self-limited as they replicate only in the target bacteria and only as long as the bacteria are present (Atterbury *et al*., 2007). Recently, due to the advantages of bacteriophages, a lot of successful research has been made in broiler chickens indicating the ability of host-specific bacteriophages alone or in combination with probiotics to reduce colonization of *Salmonella* and *Campylobacter* (Carrillo *et al.,* 2005; Atterbury*et al*., 2007; Bardina *et al.,* 2012; Marietto-Gonçalves *et al.,* 2014). It is important that both *Salmonella* and *Campylobacter* phages can be isolated from poultry feces and farm environment resulting in gut microbial stability (Atterbury*et al*., 2007). Additionally, the use of lytic bacteriophages to prevent or treat

Colibacillosis in broilers has also been studied (Lau *et al.,* 2010; Oliveira *et al.,* 2010; El Gohary *et al.,* 2014). It is worth noting that although the successful use of phage therapy in swine dates back to 1920's, it has only recently gained the attention of the research community (Zhang *et al.,* 2015). A limited number of studies on pigs indicate that the use of bacteriophages could be a successful strategy against various species of *Salmonella* (Callaway *et al.,* 2011; Albino *et al.,* 2014).

In general, a cocktail of phages that use different receptors on the host cell is more effective in reducing pathogens compared to pure phages and also delay the formation of phage resistance. Alternatively, the host ranges of phages can be genetically manipulated. More recently, molecular biology approaches have been utilized to produce recombinant phages that carry toxic genes which are expressed during phage replication. Other researchers have produced non-lytic phages that are effective at controlling the release of endotoxin due to the lack of bacterial lysis. In contrast, still others have produced permanently lytic variants of temperate phages to solve the problem of isolating phages from diverse bacterial species for which the isolation of virulent phages has been historically rare. Phage therapy holds much potential and rational design of phage therapeutics through selection, genetic engineering, and phenotypic manipulation towards host-range alteration, infection modification, and virion survival enhancement represent important components of future advancement in therapeutic efficacy (Goodridge 2010).

Bacteriophages are used not only therapeutically, but also as growth promoters in pigs and poultry (Zhao *et al.,* 2012; Wang *et al.,* 2013; Gebru *et al.,* 2014; Kim *et al.,* 2014b). Yan *et al.*(2012) suggested that a bacteriophage diet can be used as an antibiotic alternative on growth performance of pigs and in some cases bacteriophages appeared more effective than probiotics on the performance of growing pigs, as indicated by Kim *et al.* (2014). In addition, to further understanding the biology underlying phage therapy, safe practice, quality control and accumulation of knowledge and experience remain as future challenges (Chambers & Gong 2011).

It should be noted however that the oral use of probiotics or bacteriophages can be effective only if they manage to survive during the passage through the digestive system. Therefore, a successful delivery system is of utmost importance. A number of studies have been performed on poultry or swine simulated gastrointestinal conditions showing that a microencapsulation technique can protect the bacteriophages or probiotics against gastric environment (Musikasang *et al.,* 2009). In a similar study, a microencapsulated phage cocktail administered to swine feed remained effective after the passage through the GIT and successfully reduced *Salmonella* colonization (Saez *et al.,* 2011). The same results were observed in poultry with a cocktail of liposome-encapsulated bacteriophages (Colom *et al.* 2015).

However, the use of phages is still limited in controlling food borne pathogens in live animals as well as in understanding the mechanism through which they enhance animal performance. Without an understanding of the essential problems including phage resistance,

phage-host interactions, the microbial ecosystem, and the host animal, this biological pathogen control system will not be used to its fullest potential in improving swine production (Zhang *et al.,* 2015).

3.2 Section II: Prebiotics In Animal Nutrition

3.2.1 Prebiotics In Animal Feed

The deffinition, history, characteristics, types and production methods of prebiotics are the same as discussed above in Section 2.2.

3.2.2 Mode of action of prebiotics in feed

Prebiotics exert different effects on the organism and, unlike with probiotics, generalising their mode of action is not simple (IRTA 2015). Some prebiotics modulate intestinal microbial communities (increasing total aerobes bacteria and decreasing enterococci), which subsequently improved gut morphology and the epithelial brush border. Generally, the administration of prebiotics in feed directly affected the gross morphology of the intestine by promoting its development and increasing the intestinal barrier (IRTA 2015).

The chemical structure of plant cells (fibre), the limited time available for activity in certain parts of the gut and the presence of antinutritive compounds in some foods hinder the release of nutrients. Not all compounds in foods can be broken down by mammalian digestive enzymes, and so some potential nutrients are unavailable (McDonald *et al.,* 2010).

Oligosaccharides (2–20 monosaccharide units) have been claimed as beneficial nutritional modifiers for monogastric farm animals (McDonald *et al.,* 2010). They fall into the group of materials also known as prebiotics, which are defined as compounds, other than dietary nutrients, that modify the balance of the microfloral population by promoting the growth of beneficial bacteria and thereby provide a healthier intestinal environment. Oligosaccharides occur naturally in foods: soya bean meal, rapeseed meal and legumes contain galactooligosaccharides (GOS); cereals contain fructooligosaccharides (FOS); milk products have trans-galactooligosaccharides (TOS); and yeast cell walls contain mannanoligosaccharides (MOS) (McDonald *et al.,* 2010).

With respect to farm animals, prebiotics have been studied for their potential to replace

antibiotics in maintaining high feed conversion efficiencies, particularly in poultry and pork, and also to suppress methane production with ruminants (Mwenya *et al.,* 2004a,b, Sar *et al,* Santoso *et al.,* 2004 in Lee & Salaminien 2009). Regarding the manipulation of the gastrointestignal microbiota, it has been suggested that these compounds achieve their beneficial effects in the gut in two ways. First, although they can not be easily digested by the hosts' digestive enzymes, compounds such as FOS which can be used as energy substances and thereby be fermented by several intestignal microorganisms (e.g. *Bifidobacterium* and *Lactobacillus sp*.). Secondly, the gut microbial population may be altered by the oligosaccharide interfering with the attachment of harmful bacteria to the gut wall. As a means of cell recognition, all cell types have a unique configuration of carbohydrate-containing compounds (glycoproteins and glycolipids) on their surface.

Pathogenic bacterial cells have surface compounds called lectins that recognise these carbohydrates and by which they attach to the gut cells. Once attached, the bacteria are able to multiply and produce their harmful effects as soon as they came into adequate amounds. Species such as *Salmonella* and *E. coli* have a mannose-specific lectin that binds to mannose residues on the gut mucosal surface. By introducing mannose-containing compounds (MOS) into the diet, the binding by pathogenic bacteria is disrupted and instead they bind to the oligosaccharide and are carried out of the gut with the passage of the GIT (**Fig. 10**). Yeasts have mannans in the cell wall structure and form the basis of some commercial products that are claimed to act in this way. Indeed, the presence of such yeast fragments has been said to be the reason why yeast products are beneficial (McDonald *et al.,* 2010).

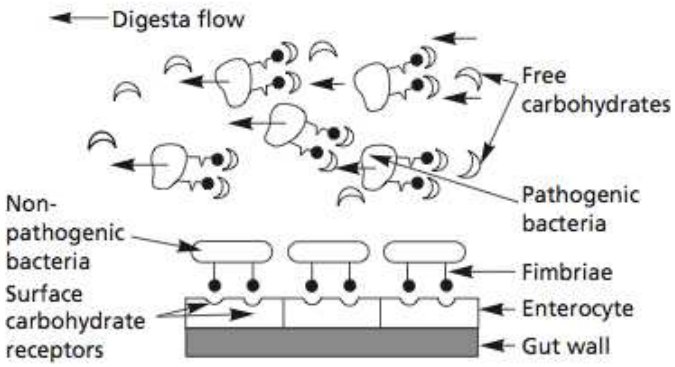

Figure 11. Adherence of pathogens on oligosaccharides.
The lectin–carbohydrate combination is specific to a particular host. However, if the same carbohydrate (e.g. an oligosaccharide) is provided in the diet, harmful bacteria can be encouraged to attach to these and they do not adhere to the gut wall but are excreted without producing toxins.(From Ewing W N and Cole D J A 1994 *The Living Gut,* Dungannon, Context in McDonald *et al.*, 2010)

The efficacy of products containing oligosaccharides is currently the subject of active

experimentation. There can be no guarantee that an oligosaccharide will favour the growth of beneficial species in a complex microflora such as that found in the pig intestine. Experiments have shown that piglets given an oral challenge of *E. coli* responded to GOS with a reduced pH of ileal digesta and reduced population of coliforms. Supplements of FOS and TOS have reduced the numbers of aerobic bacteria in the gut of weaned piglets, and there are reports of a reduced incidence of diarrhoea. Under farm conditions, improvements in gain and food conversion efficiency of the order of 4–6 per cent have been recorded. In other experiments, reduced digesta pH has been reported, but without a detectable change in the composition of the microflora, microbial metabolites or production responses. These conflicting results may have arisen because the di*et al*ready contained some oligosaccharides or because experimental conditions tend to be less stressful than those on farms (McDonald *et al.,* 2010).

Another mode of action detected using prebiotics is the enhancement of innate immune factors by up-regulating expression of complement factors and acute phase proteins. The immunomodulatory activity of prebiotics is mediated through direct interactions with PRR receptors, such as b-glucan receptors and dectin-1 receptors that are expressed on macrophages. This interaction activates signal transduction molecules, such as NF-kB, that stimulate immune cells. Saccharides may also interact with PRRs in the form of microbe associated molecular patterns (MAMPs) such as teichoic acid, peptidoglycan, glycosylated protein, or the capsular polysaccharide of bacteria, triggering an immune response (Song *et al.,* 2014). Thus, it appears that prebiotics activate the innate immune system in two ways: (1) by directly stimulating the innate immune system, or (2) by enhancing the growth of commensal microbiota (Song *et al.,* 2014, IRTA, 2015). For more information about the mechanism of action on different animal spieces the *Review of immune stimulator substances / agents that are susceptible of being used as feed additives : mode of action and identification of end-points for efficacy assessment* of 2015 of the Institut de Recerca i Tecnologia Agroalimentàries (IRTA) is suggested.

3.2.3 Positive effects on prebiotic administration in animals

Prebiotics have been trialed for use in both farm animal feeds and for companion animals. Generally, these products have beneficial effects on performance, gut structure, and the modulation of immune system (IRTA, 2015);

For polygastric animals

Similar to probiotics, prebiotics which are non-digestible oligosaccharides, are also effective in altering the composition and activity of GIT microbiome, since they constitute suitable substrates for the enhancement of certain beneficial ruminal microorganisms. However, the ability of ruminants to catabolize the most of the common prebiotics compounds, create a limitation in the use of prebiotics as growth promoters in ruminant production. In addition, several non-digestible oligosaccharides found naturally in plant cell wall are included in feeds normally used in ruminant rations (Gaggìa *et al.* 2010) making the implementation of prebiotics in ruminants possibly unnecessary. The administration of prebiotics seems to be beneficial on very young ruminants, since it contributes to the formation of a desirable intestinal community, which may further improve the performance of older animals (Uyeno *et al.,* 2015).

Despite of the wide applicability of probiotics and to a lesser extent of prebiotics in ruminant production and the promising results obtained from various studies, reproducibility issues are raised, since experimental data acquired are often inconsistent (Uyeno *et al.,* 2015). Evidently, comprehensive research is needed for the reliable and viable use of probiotics and prebiotics in ruminant production.

For monogastric animals

Although the concept of functional foods has been introduced a long time ago, scientific evidence for the use of prebiotics in animal feed exists from the late '90s for poultry and pigs (Hajati & Rezaei 2010). The majority of research in prebiotics has been performed in poultry, as this is the most studied monogastric animal. Prebiotics have a knock-on effect in immunity function in poultry, as it could be described in terms of gut development. Prebiotics were found to increase the stool volume of chicken by regulating intestinal microflora through selective stimulation of beneficial bacteria and inhibiting undesirable bacteria, such as *Salmonella* (Totton *et al.,* 2012). The most common prebiotics studied in monogastric animals is the glycan inulin, fructo-oligosaccharides (FOS) mannan-oligosaccharides (MOS), yeast cell wall (YCW)and other minor groops (IRTA, 2015). Such inconsistent results have been mainly recorded after the use of MOS to reduce the intestinal numbers of *Clostridiumperfringens* in poultry and after the use of inulin as prebiotic to improve growth performance of rabbits, layers and broilers, indicating that the effects are both dose- and diet-dependent, morphological development (villi height and goblet cell number), an increased colonisation by beneficial bacteria, and a decrease of pathogenic bacterial counts (Ortiz *et al.,* 2009, IRTA, 2015). Supplementation of poultry and porcine diets with YCW showed to improve performance and increased the height and width of the villi, which improve the absorption of nutrients. YCW may enhance the cell-mediated immune response in broiler chickens by modulating the production of both pro- and anti-inflammatory cytokines (IRTA, 2015). β-Glucan acts as an immunoprotective agent by upregulating the inflammatory response, leading to enhanced protection against intracellular pathogens (IRTA, 2015).

It is difficult, however, to draw conclusions for the prebiotic effects in animals from the published studies due to the wide variety of these studies regarding subjects, age, diet, outcome parameters, substances tested, dose, and duration of the experiments (Allaart *et al.,* 2013). The application of prebiotics in animal feed is a relatively recent effort and although the results are promising many issues must be solved, such as the establishment of the efficacy of prebiotics in routine diets of livestock. The advanced techniques like next generation sequencing could be very useful to substantiate prebiotic effect on animal microflora, while at the same time future research of prebiotics in livestock should be focused on immunological aspects, changes at gut epithelial tissues and livestock product quality.

For aquaculture

Additionally, the first study on prebiotics in aquaculture was reported in 1995 (Hanley *et al,* 1995). Despite the potential benefits of prebiotics to health and performance as noted in various terrestrial species, less information is available about the effect of prebiotics in aquatic organisms. The most common prebiotics used in aquatic species are glycan (inulin), mannan oligosaccharides (MOS), fructooligosaccharides (FOS), Yeast cell wall (YCW), and in a lesser extend short-chain fructooligosaccharides (scFOS), trans-galactooligosaccharides (TOS), galactooligosaccharides (GOS), xylooligosaccharides (XOS), arabinoxylooligosaccharides (AXOS) and various commercial products containing multiple prebiotic combinations (IRTA, 2015, Merrifield and Ringo, 2014). Prebiotic applications in aquaculture improve animal growth performance and survival, feed conversion and digestibility, gastrointestinal (GI) enzyme activities and GI morphology, as well as the suppression of potentially pathogenic bacteria due to the presence of beneficial gut bacteria (Merrifield and Ringo, 2014). Hence prebiotics play a major role in improving host health. Prebiotics are used as energy sources for the gut bacteria and can be referred to as functional saccharides or, in this respect, as immunosaccharides, which stimulate the innate immune system directly rather than via by-products of probiotics. However, it should be noted that a prebiotic is not necessarily an immunostimulant and vice versa. The role of prebiotics as immunostimulants in aquaculture is also well-studied with promising results (Song *et al.,* 2014).

One major topic that needs to be answered is whether the prebiotics supplementation effect can vary in regard to age- and size-related responses, appropriate doses and timing of administration and the life stage of the animal. Furthermore, the surrounding environment, i.e. water temperature and salinity, oxygen availability, might have greater influences than the diet on animal health or potentially confound interpretations of the prebiotic findings (Merrifield and Ringo, 2014). Further research is needed in order to differentiate the health-promoting effects from potentially deleterious responses of prebiotics.

For pets

The use of prebiotics in companion animal nutrition was reviewed comprehensively by Swanson and Faheyand their colleagues. The advantage of feeding oligosaccharides in dogs and cats is the reduction of odor and improvement in volume and consistency of feces (Swanson & Fahey, 2006; Lee & Salaminien 2009). Among prebiotics, fructo-oligosaccharides (FOS) are the most studied in dogs and cats, followed by mannanoligosaccharides (MOS), inulin and yeast wall (source of MOS among other substances) in dogs (Swanson *et al.,* 2002, IRTA, 2015). FOS have been used to alleviate small intestinal bacterial overgrowth, increase of bifidobacteria and lactobacilli populations, reduce the concentrations of protein catabolites produced in the colon (Swanson *et al.,* 2002) and tended to reduce the percentage of blood lymphocytes in cats (IRTA, 2015). As regards the effect onclostridia the effects are debatable (Swanson *et al.,* 2002,Pinna & Biagi 2014). Furthermore, Swanson and Fahey (2010)reported the immunomodulatory effect of MOS in dogs, in particular on the concentrations of IgA, IgG and plasma lymphocytes revealing a protective effect for diseased or immunocompromised animals. The amounds of the prebiotic compounds seems to be critial. One negative consequence was the tendency to reduce the apparent digestibility of dry and organic matter. Inulin in dogs resulted in increased faeces production, increased *Lactobacillus sp.*, and lower crude protein apparent in faecal digestibility. In dogs, the yeast cell wall increased *Bifidobacterium*, reduced *E. coli* counts, reduced the percentage of white blood cells in blood, and tended to increase IgA in serum and ileum (IRTA, 2015).

Molecular techniques have been also employed to evaluate the effect of prebiotics on the GIT microbial consortia in cats and dogs (Middelbos *et al*., 2007). However, further work is necessary to confirm the above results and also to elucidate the effect of prebiotics in other diseases, such as inflammatory bowel disease, small intestinal bacterial overgrowth etc.

Studies evaluating prebiotics have utilized several outcome variables to assess efficacy in canine and feline diets, including (1) food intake, (2) fecal output, (3) stool consistency, (4) macronutrient digestibility (ileal and total tract apparent digestibility), (5) fermentative end-products, (6) immune indices and (7) intestinal microbial populations. From the limited number of experiments published in this area, it appears that prebiotic supplementation has several beneficial effects in the GIT of dogs and cats, such as positive shifts in microbial populations, decreases in fecal protein catabolites and changes in immune status. However, more research is required to identify optimal doses, life stages most likely to benefit, and disease stated likely to be avoided or treated with prebiotic supplementation. In the future, experiments also must test prebiotic supplementation on animals of different life stages and disease states.

Conclusions

This thesis isaimingto investigate the latest studies and analysis of bibliography on probiotic bacteria and prebiotic carbohydrates and to analyse briefly their properties, function and role of those on the enhancement and maintainance of health.

The gut microbiota is an essential component of longevity. Current information on the microbiota and all the genes that they encode, the microbiome, represents diverse beneficial metabolic activities that may not be encoded in the hosts genome. Host and microbiota have co-envolved, because of the important role they have in nutrition and metabolism, the ability to break down indigestible carbohydrates and plant polysaccharides, in increasing the efficiency of enquiring nutrients. Metabolism is related to the immune system (IS) and *vice versa*. Gut microbiota maintain a critical position in modulating and assist in the shaping of hosts' IS.Probiotics are a variable cluster of those organisms which can be easily cultured in the laboratory and can provide health benefits similar as medicines. In turn, studies suggest that diet and nutrition can shape microbiota.A healthy, holistic, diet that includes prebiotic componenments especially with fermentable substances, such as soluble oligosaccharides, fructooligosacharides or non-soluble fibers like cellulose, inulin and resistant starch, is proven to be beneficial as growth substances for the probiotic associates.

After long-term studies, there are several claims from both independent scientists and worldwide organizations confirming through a series of studies that pro- and prebiotics play an important role in humans and animals in enhancing and promoting health.

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