Food Reviews International

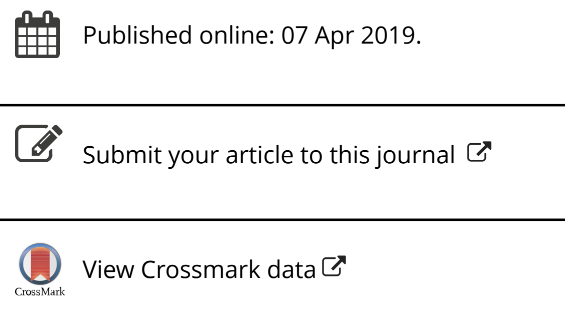
ISSN: 8755-9129 (Print) 1525-6103 (Online) Journal homepage: https://www.tandfonline.com/loi/lfri20

A Review of Criteria and Methods for Evaluating the Probiotic Potential of Microorganisms

Stellah Byakika, Ivan Muzira Mukisa, Yusuf Byenkya Byaruhanga & Charles Muyanja

To cite this article: Stellah Byakika, Ivan Muzira Mukisa, Yusuf Byenkya Byaruhanga & Charles Muyanja (2019): A Review of Criteria and Methods for Evaluating the Probiotic Potential of Microorganisms, Food Reviews International, DOI: 10.1080/87559129.2019.1584815

To link to this article: https://doi.org/10.1080/87559129.2019.1584815



Published online: 07 Apr 2019.

Submit your article to this journal

View Crossmark data

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=lfri20

FOOD REVIEWS INTERNATIONAL
https://doi.org/10.1080/87559129.2019.1584815

A Review of Criteria and Methods for Evaluating the Probiotic Potential of Microorganisms

Stellah Byakika, Ivan Muzira Mukisa, Yusuf Byenkya Byaruhanga, and Charles Muyanja

Department of Food Technology and Nutrition, School of Food Technology Nutrition and Bioengineering, College of Agricultural and Environmental Sciences, Makerere University, Kampala, Uganda

ABSTRACT

Probiotics are live organisms that, when consumed in sufficient quantities, confer health benefits to the host. There is global interest in probiotics research; many are isolated from diverse niches and identified in numerous ways. The challenge is selecting the precise range of tests and methods to use. Some tests are argued to be outdated, biased and/or irrelevant. Screening guidelines exist, but at times are not strictly followed. In many countries, firm regulations regarding probiotics declaration and use are nonexistent. Consequently, some organisms are presented as probiotics without strong scientific evidence. This review discusses common screening approaches, their relevance, strengths and weaknesses.

KEYWORDS
Probiotics; screening methods; health benefits; safety

Abbreviations: ADP: Adenosine Diphosphate; ATCC: American Type Culture Collection; ATP: Adenosine Triphosphate; BSH: Bile Salt Hydrolase; Caco-2: Cancer coli-2; cFDA-SE: Carboxyfluorescein diacetate succinimidyl ester; CLSI: Clinical and Laboratory Standards Institute; DNA: Deoxyribonucleic acid; EPN: Epinephrine; FAO: Food and Agriculture Organization; FbPA: Fibronectin binding protein A; FDA: Food and Drug Authority; GIT: Gastrointestinal tract; GRAS: Generally Recognized as Safe; HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HT-29: Human colon adenocarcinoma cells; HT-29 MTX: Methotrexate treated human colon adenocarcinoma cells; ICMR-DBT: Indian Council of Medical Research and Department of Biotechnology; IBS: Irritable Bowel Syndrome; ICR: Inherited Cataract Rat; LAB: Lactic Acid Bacteria; MFI: Mean Fluorescence Intensity; MIC: Minimum Inhibitory Concentration; mRNA: Messenger Ribonucleic Acid; MRS: de Man Rogosa Sharpe; NCCB: Netherlands Culture Collection of Bacteria; OD: Optical Density; PBS: Phosphate Buffered Saline; PGE: Prostaglandin; RBC: Red Blood Cells; SHIME: Simulator of the Human Intestinal Microbial Ecosystem; SDS-PAGE: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; TLC: Thin-Layer Chromatography; TRAP-6: Thrombin Receptor Activator Peptide-6; WHO: World Health Organization

Background

Consumers are increasingly becoming aware of the impact of diet on health. The probiotics sector is one of the fastest growing sectors of functional foods on the market. The global probiotics market was valued at USD 32.06 billion in 2013.[1] The market for

CONTACT Stellah Byakika stellahbyakika@gmail.com Department of Food Technology and Nutrition, School of Food Technology Nutrition and Bioengineering, College of Agricultural and Environmental Sciences, Makerere University, Kampala P O Box 7062, Uganda

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/lfri.

© 2019 Taylor & Francis

probiotics was later estimated at USD 33.19 billion in 2015 and is projected to reach USD 46.55 billion by 2020 and USD 64.02 billion by 2022.[2] The expansion of this market is likely due to increasing global consumer health awareness driven by the effectiveness of probiotics in preventing and treating various health conditions and growth of global functional food industry.[3,4] There is, therefore, a progressive tendency towards the consumption of functional foods because many people are more concerned about disease prevention than cure.

The functional food concept describes foods or food ingredients that exhibit beneficial effects on the health of a consumer beyond their nutritive value.[5] Functional foods include food products containing biologically active components such as probiotics, antioxidants and fiber that improve health.[6] In most countries, the largest share of functional foods is held by probiotics.[7] For instance, the food and beverage applications of probiotics in North America are estimated to increase by 50% in 2020.[1]

Attention to the health benefits associated with consumption of probiotic foods began about a century ago when intriguing observations were made among certain populations; specifically, it was reported that Bulgarians and the Russians of the Steppes lived longer than other populations possibly due to consumption of sour milk that contained beneficial bacteria.[8] The bacteria in the milk are/were believed to modify the composition of gut microflora and thus promote health. Tissier[9] of the Pasteur Institute, isolated *Bifidobacterium (B.) bifidum* from the feces of healthy breastfed infants. This bacterium was missing in the feces of formula-fed infants suffering from diarrhea. Consequently, it was recommended that *B. bifidum* be given to infants suffering from diarrhea. This followed a series of studies[10–14] on these beneficial bacteria and later established the contribution of probiotic bacteria to human health.

According to Fuller[15], 'probiotic' is a Greek term meaning 'for life' but the definition has evolved over the years. The evolution has been linked to the increasing interest in the use of viable bacterial supplements and the progress made in understanding their mechanisms of action. Initially, the term defined substances produced by an organism that stimulated the growth of other organisms. Later, it was used to describe tissue extracts that stimulated the growth of microorganisms, and animal feed supplements that had a beneficial effect on the intestinal microflora. Fuller[16] defined probiotics as 'live microbial feed supplements which beneficially affect the host animal in improving microbial balance.' This definition was later modified by FAO/WHO[17] to mean 'live microorganisms which when administered in adequate amounts confer health benefits on the host.' A key property for a probiotic microorganism is colonization of the gut once the minimum dose and timing (period of consumption) is achieved, assuming the consumer is not on antibiotics. According to Cunha et al.[18], the beneficial effect of a probiotic to its host is related to its concentration in the intestinal lumen and its value must be at least 10^7 cfu/g of fecal content. On the other hand, a probiotic food should have a minimum of 10^6 cfu/g or mL and a total of 10^8 to 10^9 probiotic microorganisms should be consumed daily for the probiotic effect to be transferred to the consumer.[5] According to Minelli and Benini[19], the usual effective dosage in humans is 10^7–10^9 cfu/mg per day. However, Sanders[20] states that the adequate amounts of probiotics should be based on efficacious levels in human studies because one dose level cannot be assumed effective for all strains. For instance, the efficacy of *B. infantis* 35264 is 10^8 cfu/day[21], for VSL#3 (a combination of freeze-dried lactic acid bacteria and *Bifidobacteria*) is 10^{12} cfu/day[22] and for

Lactobacillus (Lb.) reuteri Protectis is 10^7 cfu/day.[23] Therefore, one of the major knowledge gaps that necessitate further inquiry is the minimum dose and/or frequency of consumption of probiotics for beneficial effect.

Increasing health-care costs, preference for preventive therapies for chronic illnesses and natural therapies will continue to drive probiotic market growth. The new market research report on probiotics stated that Japan, Europe and the United States represent the major markets for probiotics worldwide.[4] Aging populations, leading probiotic manufacturers, high spending power of consumers, focus on preventive health-care and the desire to maintain active lifestyles are some of the key factors driving growth of probiotics in Japan, Europe and the United States.[4] In the Asia-Pacific particularly, the key growth drivers include the expanding middle-class population with high disposable income, aggressive retail marketing, westernization of food habits and robust production and consumption of functional foods.

Africa and the Middle East are the latest potential markets for probiotics and are showing exceptional growth in the consumption of these products.[4] These regions are predicted to have continued growth and will be the future epicenters of global probiotics sales by 2020.[24] Information on the market share of probiotics in Africa is limited and is likely to be quite small on the global scale.[3] In Africa, South Africa has a slightly well/more established market for probiotics; however, when compared to, for example, the European countries, the market is still underdeveloped and unpenetrated. One major challenge in marketing probiotics especially in developing countries is lack of awareness and their premium price. The Global Probiotics Market 2017–2021 report[25] illustrates this by comparing the price of probiotic yogurt ($2.4 per ounce) to regular yogurt ($1 per ounce). The premium price is mainly attributed to patents and use of co-cultures. Moreover, in countries such as India where yogurt is homemade, selling probiotic yogurt at a premium price just cripples its market penetration because consumers will most likely not be interested in an overpriced new product. Interestingly, it is known from indigenous knowledge and practices that African traditional fermented products are used in prevention and treatment of gastrointestinal tract (GIT) illnesses.[26] There is also increasing interest in studying the probiotic properties of African traditional fermented foods and probiotic isolates from these products.[26–29]

Increasing interest in probiotics worldwide is responsible for the efforts towards isolating probiotic organisms from local traditional products.[27–29] Several tests have to be carried out on candidate microorganisms before they can be confirmed and accepted for use as probiotics. The importance of these tests generally lies in ensuring efficacy and safety for the consumer. Various criteria and methods have been suggested and applied. Although the FAO/WHO summarized the criteria to be used, it does not specify which methods should be used for the different criteria. Different methods may vary in ease of use, resolution, cost and interpretation. The advance effects of using unsuitable, irrelevant and nonexhaustive methods/protocols may include declaring unsafe and inefficacious organisms as probiotics. This could not only negatively impact on consumer health but is also a recipe for fraudulent trade in the probiotics market. Therefore, this paper reviews the criteria and methods employed in the evaluation of probiotic activity of candidate strains. It highlights the pros and cons of different methods and offers recommendations regarding the screening process.

Probiotic organisms

The most common probiotic microorganisms are *Bifidobacteria* and lactic acid bacteria (LAB) belonging to the genera *Lactobacillus*.[30] Other less common probiotic organisms belong to the bacterial genera *Streptococcus, Propionibacterium, Bacillus, Enterococcus* and *Escherichia*[31–34] and the yeast genus *Saccharomyces*.[35]

Bacillus is a less known genus of probiotics as the majority, notably *Bacillus cereus* and *Bacillus anthracis* are pathogenic. Fortunately, their pathogenicity is strain specific since some strains do not produce enterotoxins and can thus be used as probiotics.[36] Production of endospores by *Bacilli* enables them to survive the harsh gastric conditions[37] and allows for storage of the probiotics at room temperature and in dried form.[38]

Enterococci are also used as probiotics although this genus is not considered 'Generally Recognized as Safe (GRAS)' because some strains, notably *Enterococcus faecalis* and *Enterococcus faecium*, have been implicated in opportunistic infections.[39,40] Nevertheless, the genus has been and is still a target for probiotic studies[41–43] because some strains have desirable properties such as the production of enterocin, which is an important bacteriocin.[41]

Escherichia (E.) coli is another rare example of a non-LAB probiotic. In 1917, during World War I in Germany, Alfred Nissle a physician and bacteriologist, made an amazing discovery about a probiotic bacterium during an outbreak of shigellosis among the soldiers. Interestingly, one of the soldiers did not succumb to shigellosis. Nissle isolated a bacterium (*E. coli* Nissle 1971) from the feces of the soldier who did not suffer from shigellosis and successfully used it to treat other soldiers. *E. coli* Nissle (EcN1917) is now an active component of the pharmaceutical preparation 'Mutaflor®', which is a licensed microbial drug used in the treatment of intestinal infections in Germany and some European countries.[44,45] *E. coli* M-17 is another rare example of a non-LAB beneficial microorganism that is not regulated by the FDA but is considered merely as a health food supplement. The organism was discovered in Russia and was widely used in former Soviet Union countries to treat GIT ailments. It continues to be produced and marketed under government control in the Russian Federation.[46]

Although most probiotic organisms are bacteria, a probiotic yeast strain known as *Saccharomyces boulardii* also exists. This strain is effective in the prevention of antibiotic-associated diarrhea.[47]

Various health benefits associated with probiotic organisms have been extensively discussed.[5,11,35,48–55] The benefits include: lifespan prolongation, production of antioxidants and other geroprotectors, inhibition of pathogens in the GIT, relief from lactose intolerance, reduction in serum cholesterol, improved immune response, prevention of cancers, treatment and prevention of ulcerative colitis, and atopic dermatitis, treatment of IBS, reduction in cardiovascular disease risk factors, treatment of *Helicobacter pyroli*, prevention of antibiotic-associated diarrhea, production of vitamins, treatment and prevention of urogenital infections and hyperoxaluria among others. Table 1 summarizes the commercial probiotic microorganisms, origins, carrier products and their health benefits.

Table 1. Selected commercial probiotic microorganisms, origins, carrier products and health benefits.

Product/Company	Probiotic microbe	Carrier product	Health benefit	Reference for health benefit
Yoba for Life Foundation, The Netherlands and Chr. Hansen Holding A/S, Denmark.	*Lb. rhamnosus* GG	Dairy, infant formula, dietary suppl.	Alleviation of rota virus diarrhea, prevention of travellors' diarrhea and *Clostridum difficile* colitis, prevents atopic dermatitis, improves symptoms of IBS, immunomodulation.	[56,57]
Probi AB, Sweden.	*Lb. plantarum* 299 v	Dairy, fruit drinks, dietary suppl.	Improves symptoms of irritable bowel syndrome, increases iron absorption.	[58]
Yakult® -Yakult Honsha Co. Ltd, Japan.	*Lb. casei* Shirota	Dairy.	Intestinal flora reposition, improved digestion.	[59]
Align® – Procter and Gamble, U.S.A.	*B. infantis* 35624	Dietary suppl.	Immunomodulation, maintains digestive balance.	[60]
Bioflorin -Cerbios Pharma, Switzerland.	*En. faecum* LABSF 68	Hard gelatin capsules.	Prevention and treatment of intestinal disorders.	[61]
Mutaflor® (Ardeypharm), Germany.	*E. coli* Nissle 1917.	Dietary suppl.	Pathogen inhibition, treatment of inflammatory bowel and functional bowel disease.	[62]
Ginophilus® (Probionov), France.	*Lb. casei* rhamnosus Lcr 35	Capsule	Restoration of normal vaginal flora after antibiotic treatment of bacterial vaginosis, treatment of chronic constipation.	[63, 64]
Activia® Yogurt (Danone), France.	*B. animalis lactis* DN-173 010/CNCM I-2494	Yogurt	May prevent disorders associated with increased gut barrier permeability, regulate digestive system.	[65]
SVELTY® Gastro Protect (Nestlé), Switzerland.	*Lb. johnsonii* La1	Fermented milk.	Controls *H. pylori* infection and stomach discomfort.	[66]
LC1 Yogurt® (Nestlé), Switzerland.	*Lb. johnsonii* La1 and *Lb. cidophilus*	Yogurt.	Control of *H. pylori* infection and stomach discomfort	[66]
Actimel® (Danone), France.	*Lb. casei* defensis DN-114001	Milk.	Reduction of prevalence of antibiotic associated diarrhea.	[67]
Yomogi® 250 mg, Germany.	*S. boulardii* (*S. cerevisiae* HANSEN CBS 5926)	Hard capsules.	Treatment against acute and traveler's diarrhea.	[68]
HOWARU® Protect Probiotics (Danisco A/S), Denmark.	*B. lactis* Bi-07, *Lb. acidophilus* NCFM, *B. lactis* B1-04	Dietary suppl.	Improve symptoms of functional bowel disorders.	[69]
Paidoflor®, Germany.	*Lb. acidophilus*	Chewable tablet.	Restores balance and GIT regularity during diarrhea and obstipation.	[70]
Sunny Crunch Foods Ltd, Probiotic Chewy Cereal Bars, Canada.	*B. coagulans* GBI-30, 6086	Cereal bars.	Improves protein absorption and utilization and immune health.	[71]
PROBIO-TEC® (Chr. Hansen A/S), Denmark.	*Lb. acidophilus* LA-5 and *B.* BB-12	Infant formula, dietary suppl.	Prevent gastrointestinal and lower respiratory tract infections, support the re-colonization of the intestinal microbiota.	[72]

Lb.; Lactobacillus, B.; Bifidobacterium, L. Lactococcus, St.; Streptococcus, S.; Saccharomyces, En.; Enterococcus, Suppl.; Supplement, IBS; Irritable Bowel Syndrome.

Probiotic benefits can be demonstrated using *in vitro* and *in vivo* methods. Although many probiotic benefits have been shown using *in vitro* methods, it is mandatory for these to be validated *in vivo*, and most specifically through human clinical trials.[73]

Current guidelines for evaluating candidate probiotic organisms

There were generally no agreed upon standard criteria for studying probiotics prior to the establishment of the guidelines for evaluation and declaration of probiotics for food use by the Joint FAO/WHO Working Group.[73] Development of these guidelines was intended to streamline the evaluation process and ensure not only the safety of probiotics but also the authenticity of the health benefit claims associated with each organism. Although these guidelines are available, the drawback is that they do not recommend specific *in vitro* and *in vivo* techniques for use in evaluating probiotic properties. The other concern is that these guidelines are not always strictly adhered to. This is possibly due to lack of enforcement by regulatory bodies in some countries, which is in turn a result of limited knowledge, infrastructural capacity, testing protocols and resources. Consequently, the market is flooded with numerous so-called probiotics whose authenticity cannot be defended.[74]

Following the increased influx of probiotic products on the Indian market, and lack of a systematic approach to evaluate the probiotics in food for their safety and efficacy, the Indian Council of Medical Research (ICMR) and Department of Biotechnology (DBT) formulated relevant guidelines for use in evaluating probiotics.[75] These guidelines were approved for use in India. The guidelines (summarized in Figure 1) define the parameters required for a product or strain to be termed 'probiotic.' The ICMR-DBT taskforce took into consideration guidelines by the Joint FAO/WHO Working Group[73] and a few other related sources.[75] The ICMR-DBT guidelines for evaluation of probiotics in food appear to be a summarized version of those previously developed by the Joint FAO/WHO Working Group.[73] Furthermore, the key difference is that according to the ICMR-DBT guidelines a microorganism can only be considered probiotic after undergoing the three phases (safety, efficacy and effectiveness) of human clinical trials while the third phase (effectiveness) appears to be optional in the Joint FAO/WHO Working Group[73] guidelines. We contend that while it is beneficial to compare a probiotic organism to the standard treatment of a specific condition (phase III: effectiveness), both phase I (safety) and phase II (efficacy) human clinical trials are sufficient to meet the requirements for defining a probiotic organism.[17]

Probiotic studies generally involve three major components: determining their survival in the GIT, safety for human or animal use, and establishing probiotic activity/benefit to the consumer. These health benefits are strain specific and do not necessarily cut across all probiotic organisms or even members of the same species.[76]

In vitro and *in vivo* tests are employed in the characterization of probiotic strains. *In vitro* tests are generally much easier to perform but may not fully predict the functionality of probiotic organisms in the human body.[73] Therefore, *in vitro* data alone is insufficient for describing an organism as probiotic. In addition, when screening for safety, virulence of a candidate strain may be inactive under the specific conditions of the *in vitro* assay.[77] Papadimitriou et al.[77] argue that virulence is a complex phenomenon that at times needs active interaction with the host to be triggered, thus suggesting that *in vivo* models are more appropriate during microbial safety investigations. Nonetheless, even though *in vivo*

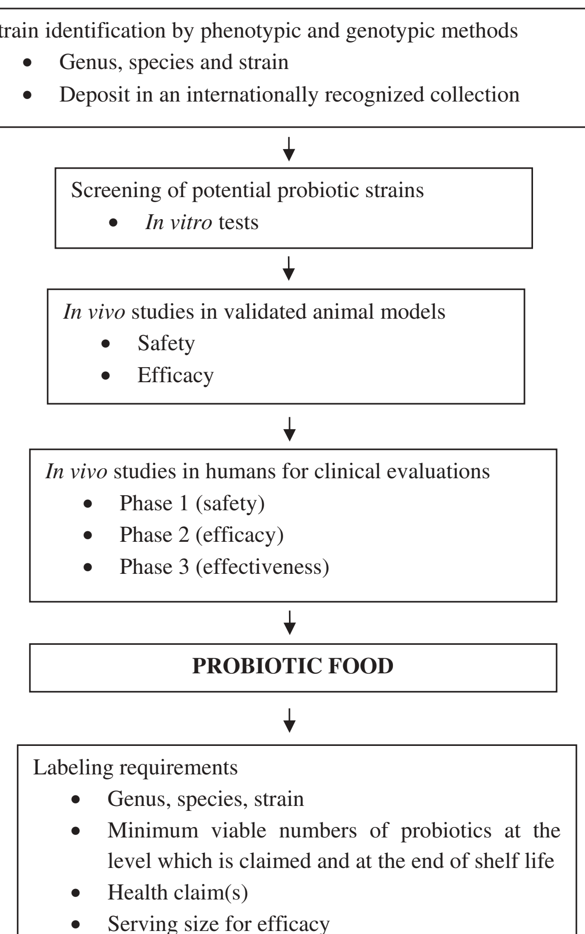


Figure 1. Guidelines for evaluation of candidate probiotics strains. (Figure reproduced with permission from ICMR-DBT guidelines for evaluation of probiotics in food. 2011, Vol. 134, Issue no. 1, 22–25, Ganguly et al. Copyright: The Indian Journal of Medical Research.)

tests are more appropriate, many of them cannot be used repetitively for multiple strains due to financial and ethical reasons.

The Joint FAO/WHO Working Group[73] states that probiotics for human use should be substantiated for efficacy, first using animal models followed by human trials. There are countless tests that can be employed in the characterization of probiotic organisms but the following, according to the Joint FAO/WHO Working Group[73], are the major ones:

(1) Resistance to gastric acidity,
(2) Bile acid resistance,
(3) Adherence to mucus and/or human epithelial cells and cell lines,
(4) Antimicrobial activity against potentially pathogenic bacteria,
(5) Ability to reduce pathogen adhesion to surfaces and

(6) Bile Salt Hydrolase (BSH) activity.

Apart from adherence to mucus and/or human epithelial cells and cell lines, the above listed major tests are exactly the same as the ICMR-DBT guidelines for *in vitro* characterization of probiotic strains.[75]

In as far as the organism safety is concerned, the Joint FAO/WHO Working Group[73] recommends at a minimum the following screening tests:

(1) Antibiotic resistance patterns,
(2) Metabolic activities such as D-lactate production, bile salt deconjugation, etc.,
(3) Assessing for side effects in humans,
(4) Epidemiological surveillance of adverse incidents in consumers,
(5) Toxin production (for strains that belong to a species known for toxin production) and
(6) Hemolytic activity for strains that belong to a species known for hemolysis.

The ICMR-DBT guidelines[75] recommend the following tests for evaluation of microbial safety:

(1) Determining antibiotic resistance,
(2) Checking for undesirable side-effects,
(3) Testing for toxin production and hemolytic activity especially for strains belonging to a species known to produce toxin or to have hemolytic potential and
(4) Establishing that the candidate strain does not cause infections in immunocompromised individuals.

In spite of the obvious need to establish the safety of a candidate organism, this should only be strict for species (for instance *Bacilli, Enterococci* and *E. coli*) with a known history of pathogenicity. The safety of species such as lactobacilli and bifidobacteria, which are historically associated with food and considered safe for human consumption[78], should not be worrisome. However, Marteau[79] and Snydman[80] argue that, despite their GRAS status and health benefits, probiotic organisms are still a safety concern because theoretically they can cause: (1) systemic infections such as bacteremia or endocarditis, (2) toxic metabolic activities in the GIT, (3) excessive immune stimulation in susceptible individuals and (4) gene transfer, particularly antibiotic resistance genes to gastrointestinal flora. Even though these could be a possibility, there is still no plausible evidence to support these claims. For instance, in infections where probiotic organisms have been isolated, they mostly occur as co-isolates without evidence of a primary role in the infection.[78] The organisms appear to originate from the host's own microflora.[78] Furthermore, Adams and Marteau[78] reported no cases of infection in people working with or routinely exposed to high probiotic strain counts such as those involved in large-scale production of these cultures. Although it could be argued that workers in such facilities handle these strains with extreme caution, the absence of reports on probiotic-related infections in these facilities could also indicate that the probiotic strains are noninfective.

It could be argued that some of the traditional screening assays are very slow and outdated but they are still employed because of their simplicity, reproducibility and low

cost. Consequently, to speed up probiotics research, various new molecular omics tests based on the identification of specific molecular markers are now available.[77,81] Given their rapid nature and high accuracy, these new techniques could possibly replace traditional *in vitro* and *in vivo* tests. Omics assays not only address the limitations associated with traditional assays but the knowledge obtained from this new technology will also be used to design more simple, accurate and precise *in vitro* and *in vivo* assays.[77]

According to the Joint FAO/WHO Working Group[73], evidence supporting probiotic activity of a strain should be published in peer-reviewed scientific or medical journals. Publication of negative results is also encouraged as these contribute to the totality of the evidence to support probiotic efficacy.

Determination of gastric survival of probiotics

Tolerance to the harsh acidic and bile conditions of the GIT is vital for the selection of probiotic microorganisms.[82] The Joint FAO/WHO Working Group[73] recommends the following tests for screening of gastric survival of candidate strains: resistance to gastric acidity, bile salt tolerance and BSH activity. This section discusses these tests in detail. Other related tests are summarized in Table 2.

Tolerance to acid and bile salts

Many of the methods used for these assessments are *in vitro* because of the high costs, ethical implications and safety issues associated with *in vivo* studies.[90] However, Papadimitriou et al.[77] criticized *in vitro* acid tolerant assays. They point out that after ingestion of food, the stomach pH raises from 1–2 to 4–5, yet the majority of the *in vitro* assays are designed to select for strains that can tolerate extreme low pH (pH 2–3). This could exclude some strains that may actually possess probiotic properties but cannot tolerate extremely low acidity. While tolerance to low gastric pH conditions is vital in

Table 2. Other tests for evaluating potential probiotic strains.

Test	Rationale	Reference
Gastric survival test		
Lysozyme tolerance	Lysozyme destroys some types of microorganisms in the mouth.	[83]
Phenol tolerance	Bacterial deamination of amino acids in the gut may form phenol which is bacteriostatic against some probiotic strains.	[84]
Probiotic activity test		
β-Galactosidase activity	β-Galactosidase is essential in the reduction of lactose intolerance.	[85]
Glutathione test	Glutathione forms part of the cell defense system against oxidative stress which is important in the intestinal microbial ecosystem.	[86]
Total Antioxidative Activity (TAA)	TAA protects cells against oxidative damage. This could be due to action of different enzymatic antioxidants.	[55, 87]
Anticancer activity	Important in prevention of certain cancers for example colon cancer.	[88]
Cholesterol reduction	Hypercholesterolemia is a risk factor in coronary heart disease.	[87]
Immunomodulation	Some strains activate a host's defenses against pathogens but unrestricted immunostimulation in autoimmune illnesses is detrimental.	[89]
Safety test		
Virulence genes	Some genera such as *Enterococci* and *Bacilli* are known to possess virulence genes.	[36, 41]

screening for probiotics, we argue that it should not necessarily hinder further screening tests. With advancements in technology, such probiotics could be shielded from the harsh gastric conditions, for example, through encapsulation, subsequently allowing them to germinate and attach onto the ileum lining. Hou et al.[91] reported that the viability of *Lb. delbrueckii* ssp. *bulgaricus* in high acid and bile salt conditions was elevated by close to 4 log units after encapsulation within artificial sesame oil emulsions. Therefore, traditional acid tolerance tests could be modified to also investigate microbial survival at slightly higher pH ranges (pH = 4–5) as is the situation post ingestion of food. Furthermore, these assays are often carried out using organisms in either their log or stationary phase. This does not necessarily guarantee results similar to those obtained using organisms that have been stressed by different food processing and storage conditions.[77]

During digestion, acidified chyme moves from the stomach directly to the duodenum where it is exposed to bile salts. However, some of the *in vitro* assays separate the acid and bile treatments, yet they are sequential *in vivo*. It is more realistic to study the survival of candidate strains in the successive stress conditions.

Despite *in vitro* tests being almost inevitable, it is noteworthy that bile tolerance of strains in both systems may not reflect the actual ability to tolerate bile *in vivo*.[92] Conditions such as pH, temperature and atmosphere among others exposed to a strain prior to entry into the ileum may influence its bile tolerance level.[92] In addition, bile concentrations in the body fluctuate, they are usually very low until a fatty meal is consumed, and so pre-exposure to low bile concentrations could increase tolerance. For example, sodium cholate and sodium deoxycholate caused an extremely rapid killing of about 4 log unit reduction of *Enterococcus faecalis* ATCC 19433 exposed to 0.3% (w/v) for 15 s. However, when exposed to 0.08% (w/v) of the same bile salts for 5 s (flash adaptation), the organism was able to adapt to this low bile concentration and a substantial protection against the normally lethal levels was observed.[93] It is possible that low levels of bile rapidly intercalate with microbial membrane lipids making the resultant mixed membranes resistant to further detergent effects of the bile salts.[92] Unfortunately, the fluctuation in bile salt levels in the ileum is not incorporated in *in vitro* studies. Furthermore, *in vitro* studies expose candidate strains directly to bile salts which are contained in just (phosphate buffered saline) PBS or a broth. The presence of food in the intestine is thought to aid the survival of strains by shielding them in the food matrix; some foods may even bind the bile salts and inhibit their toxicity.[92] For instance, Shimakawa et al.[94] observed a lower inhibitory effect of bile on *B. breve* Yakult in the presence of soy proteins, which bound and aggregated the bile salts. Begley et al.[92] also argued that *in vitro* assays may exaggerate findings because bile salts in the body that are complexed in micelles with phospholipids may not be available to interact with microbial cells, thus lowering their antimicrobial activity.

The type and structure of bile is an important factor in its antimicrobial action. Deconjugated bile salts have a stronger antimicrobial effect than conjugated bile salts, and the bactericidal effect of dihydroxyl bile salts is greater than that of trihydroxyl bile salts.[95] Bovine (oxgall) and porcine are the two commercially available bile types at the time of this review. Literature reveals that of the two, bovine bile is more often used in *in vitro* assays. However, it contains trihydroxylconjugated bile salts, which have a lower antimicrobial effect than porcine bile, which not only contains dihydroxylconjugated bile salts[96] but is also more biochemically similar to human bile.[97]

There are also some irregularities in the concentrations of bile salts used in *in vitro* bile tolerance assays. According to Barrett et al.[98], the concentration of bile salts in the human hepatic duct bile is 0.7%. On the contrary, Jose et al.[99] reported 0.3% bile salt as the maximum that can be found in an average healthy person. Thus, in their study Jose et al.[99] used 0.3–2% bile salts for screening the isolates. On the other hand, according to Goldin and Gorbach[100], the tolerance to bile salt concentrations of 0.15–0.3% is recommended for probiotics, since it is in the range of the physiological concentrations met in the GIT. Therefore, considering these variations among authors, we propose a bile salts concentration range of 0.1–0.7% in bile tolerance studies.

There are several approaches for evaluating the stability of microbial strains to high acid and bile conditions. These include the tube/well method, diffusion methods, bioreactors, animal studies and omics techniques.

Tube/well method. The commonest, simplest and cheapest *in vitro* method for acid tolerance testing involves adjustment of the pH of a respective broth or PBS to different levels (pH = 1.5–3.5) using concentrated HCl. The acidified broth or PBS is inoculated with a known concentration of the bacteria (10^6–10^9 cfu/mL) and incubated for a given period of time (2–4 h). This incubation period is similar to the time food takes in the stomach before it is moved to the duodenum.[101] Samples are drawn at intervals to determine the viable cell counts or cell growth using the plating method or optical density (OD).[76,102,103] To determine acid tolerance, Kõll et al.[83] used flat-bottom microwell plates each with 180 µL MRS broth was adjusted to a pH range of 1.5–3.5 using 6 mol/l HCl. Each well was inoculated with 20 µL of overnight culture and incubated aerobically at 37°C for 4 h. Viable counts can be obtained using the plating method. To test for bile salt tolerance, Kõll et al.[83] used MRS broth containing 0.08–5.0% (v/v) human bile. A 180 µL volume of the bile adjusted MRS broth was inoculated with 20 µL of overnight culture and incubated 37°C for 24 h. survival of culture was measured at OD_{630} at 0, 3, 6, 9, 12 and 24 h. While some candidate strains may be able to survive in bile conditions for 24 h, it should be noted that chyme spends on average 4–8 h in the ileum.[101] Assays that expose candidate strains longer than 8 h could result in them dying off and considered unable to survive in the GIT.

Gastric juice or intestinal fluid of human[103–104], or animal origin or prepared synthetically[105] can also be used. As earlier stated, bovine (oxgall) and porcine bile salts are commercially available. There are different ways of obtaining human gastric juice and bile. Xanthopoulos et al.[103] obtained gastric juice from a volunteer after at least a 4 h fast by aspiration through a nanogastric tube. Their study indicates no additional treatment to the gastric juice before use. Dunne et al.[104] used laparoscopic cholecystectomy to obtain human bile. The bile was filter-sterilized through a 0.45 µm membrane before use. Laparoscopic cholecystectomy is a procedure that involves removal of the gall bladder using instruments placed into small incisions in the abdomen.[83] It is commonly employed in the treatment for patients with symptomatic gall bladder disease.[106]

Although quite popular and relatively simple to perform, tube/well methods can be laborious and inaccurate since some setups involve a lot of reagent preparations and adjustments increasing the chances of introducing human errors. One ought to be very careful and accurate in setting up the experiment because even a slight deviation in target pH and/or bile concentration(s) could completely change outcomes. Regarding acid and

bile choices, the best option would be to use those of human origin but this may require ethical approval, which in some cases is very time-consuming. Therefore, the next best option is porcine bile since it is more biochemically similar to human bile than bovine bile[97] Nevertheless, bovine bile has also been successfully used by many authors.-[26,29,34,86,107–108]

Diffusion methods. The agar well-diffusion technique can also be used to determine the bile tolerance of organisms. Briefly, Vinderola et al.[109] mixed 20 mL of MRS agar, melted and tempered to 45°C with 200 µL of cell cultures grown overnight. Wells of 10 mm diameter were made in the agar, 180 µL of bile salts solution of 0.0–1.0 g/100 mL were added to the wells. Plates were incubated overnight at 37°C, and aerobiosis and diameters of inhibition halos recorded. Todorov et al.[110] used microtitre plates, where each plate was filled with 180 µL of bile containing medium and inoculated with 20 µL culture (OD_{600nm} = 0.3). OD readings at 600 nm were recorded every hour for 10 h. Agar plates of the respective growth media containing bile salts of varying concentrations can also be used. The bacteria are either streaked[111] or spotted[112] on the agar surface and incubated. Diffusion-based methods are very simple but there is always the risk of the agar density and volume affecting the diffusion rate and extent. It is, therefore, important to ensure that a uniform amount of medium is used for all the plates used. Additionally, in diffusion-based methods, the concentration of bile salts diffused into the medium cannot be quantified.

Bioreactors. A more realistic simulation of the gastrointestinal conditions is a modification of the tube method. The modified approach involves the use of agitated flasks that simulate the acidic and gastric conditions followed by estimation of surviving cells over time.[113] The Simulator of the Human Intestinal Microbial Ecosystem (SHIME), made of about five serially connected pH-controlled bioreactors, is an even much better modification.[114] However, it is not only complex but also requires complete anaerobic conditions; moreover, the absorption of metabolites and water is not simulated.[90] Marteau et al.[115] overcame this limitation by using dialysis membranes. In their study, Marteau and colleagues [115] used a dynamic gastrointestinal model in which the jejunal and ileal compartments were equipped with hollow fiber devices to permit dialysis of the chyme.

Botes et al.[34] used a gastrointestinal model to study the acid and bile tolerance of *Lb. plantarum* 423. The model simulated the nutrient flow through the GIT of infants. The model had a 'stomach', 'duodenum', 'jejunum' and 'ileum.' At specific times, saliva, pancreatic juice and bile salts were separately pumped into the 'stomach' (pH = 3.7), 'duodenum' (pH = 6.5), 'jejunum' (pH = 6.5) and 'ileum' (pH = 6.0). Counts were determined using the plating method at 6, 8, 10, 12, 15, 18 and 21 h after inoculation with culture.

Ritter et al.[90] studied acid and bile resistance using *Lactobacillus gasseri* K7 and selected bifidobacteria using a single reactor system. Figure 2 summarizes the simulation using the bioreactor. Samples were drawn hourly to determine bacterial survival.

In this simulation, the software package was excellent at controlling the entire process.-[90] The growth media was supplemented with skim milk, which functioned as a simulated food matrix. Food matrices are reported to help microorganisms pass through the stomach and reach the intestines in high numbers.[116] The challenge with this single reactor system simulation is that while the addition of pancreatic solution and bile salts

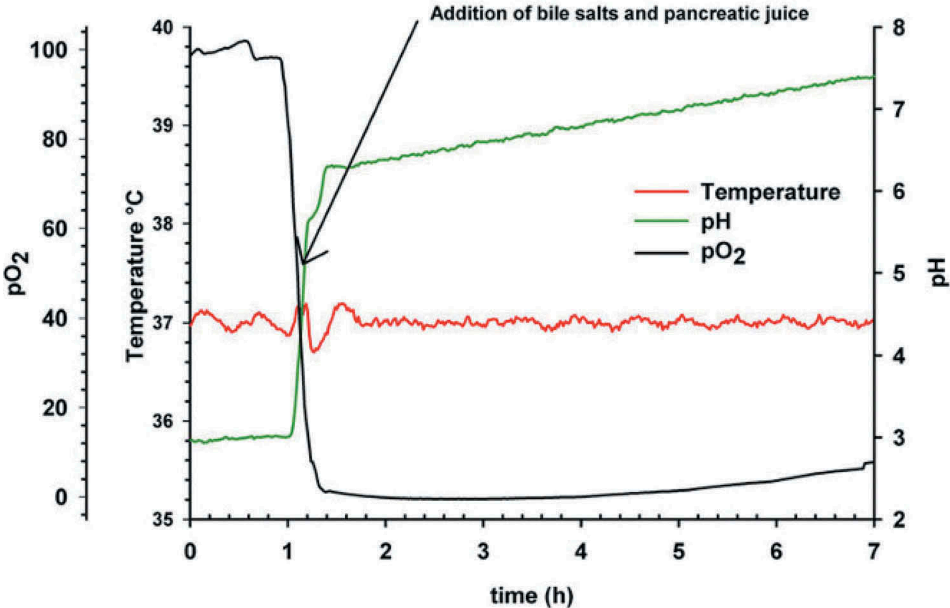


Figure 2. Parameters of the stomach-intestinal passage simulation using a bioreactor. (Source: Ritter et al.[90].)

complete the passage into the ileum, it does not consider the fact that *in vivo*, the digestive enzymes are activated and inactivated and the bile salts reabsorbed. Therefore, it does not exactly simulate the environment in the colon, creates additional stress on the bacteria and could possibly explain the unexpected linear decrease in *B. adolescentis* during the entire 7 h simulation in a similar study.[90] This is because the gastric juice and bile salt concentrations remained at the initial level and were not diluted as would be the case *in vivo*.[90] Dilution of the reactor content with a medium is reported to successfully counter this challenge. For instance, Sumeri et al.[117] used a dilution medium to simulate the decrease of bile salt concentration by absorption in the jejunum and ileum. De Boever and Verstraete[118] reported that calcium salts can be also used to precipitate bile salts at the end of the simulation to achieve a similar effect as the dilution medium. The single reactor system can reliably be used to estimate the effects of acid and bile on an organism *in vitro* before applying *in vivo* methods, which are more costly.[90]

GIT simulators are reliable and more accurate in selecting low acid and bile tolerant probiotic strains; unfortunately, they do not allow for quick screening of numerous strains and could be rather costly to operate and maintain.[77] They, however, offer better opportunities for simulation of GIT conditions and passage when compared to the tube and diffusion methods.

Animal studies. Animal models can also be used to study the survival of candidate probiotic strains to gastric conditions. For instance, *in vivo* studies involving mice have been reported. Duangjitcharoen et al.[119] labeled *Lb. plantarum* SS2 with a fluorescent dye (cFDA-SE) and fed it to ICR mice. Their feces were collected daily for two weeks and *Lb. plantarum* SS2 counts determined. Thereafter, the mice were sacrificed and their intestines

analyzed. The mucosa surface was scraped to determine the number of *Lb. plantarum* SS2 that were attached. This approach is more accurate in studying the strain survival in low pH and bile conditions of the stomach and duodenum, respectively. The method also assesses the ability of the organisms to attach onto the epithelium. There was no interference with the inherent gut LAB since the test microorganism was labeled. The limitation is the different items involved, which could be costly to acquire (for instance, the fluorescent labeling dye and mice). It may also be necessary to obtain research approval to experiment with animals and in some countries, the approval process is very time-consuming. Additionally, unlike most *in vitro* studies which require only 24–48 h, this approach requires up to two weeks, which is much longer. For studies that involve more than one strain, this method may not be only too costly but also time-consuming. In such cases, we suggest not to use this approach for initial screening but rather for in-depth studies of strains that showed relatively good survival in the *in vitro* tests. This approach provides the opportunity to use a more natural system that probiotic organisms encounter in the human GIT when compared to the tube, diffusion and GIT simulator. It also provides the opportunity for the researcher to evaluate the attachment of study strains, which is not possible with other techniques.

Omics techniques. Omics techniques are more recent methods of detecting the ability of candidate probiotic strains to survive gastric conditions. These techniques involve the holistic view of molecules that make up a cell, tissue or organism. The basic aspect of omics techniques is that a complex system is better understood when considered as a whole.[120] Omics techniques are primarily aimed at the universal detection of genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics).[120]

In reference to gastric survival, stress genes may be activated leading production of specific proteins in some microorganisms. Omics techniques can, therefore, be used to detect expression of such genes and/or proteins in stressed microorganisms.[77] For instance, heat shock proteins that repair acid damaged proteins were reported in acid-stressed organisms.[121] In addition, genes implicated in DNA repair were up-regulated in acid-stressed microorganisms.[122] Furthermore, An et al.[123] reported an up-regulation of BSH encoding genes in bile-stressed organisms. Cell permeases were also up-regulated during bile stress to expel the bile salts from the cell.[123] Koskenniemi et al.[124] reported a significant increase in transcript levels of 316 genes and 42 intracellular and surface-exposed proteins in bile stressed *Lb. rhamnosus* GG. The identified proteins suggested diverse and specific changes in general stress responses as well as cell envelope-related functions, likely for enhancing protection against bile stress. A protein dedicated to active removal of bile compounds from the cell and BSH enzyme was also up-regulated. It was concluded that the observed changes in gene expression were likely linked to pathways that enhance adaptation of the strain to bile stress. In another study, Hamon et al.[125] investigated biomarkers for *in vitro* bile tolerance in six *Lb. casei* strains. Twelve proteins associated with membrane modification, cell protection, detoxification and central metabolism were detected. These proteins were possibly key determinants of bile tolerance of *Lb. casei* and may serve as biomarkers for screening.[125]

While a lot of research has been focused on identifying specific stress genes and proteins[121–125], Papadimitriou et al.[77] argue that many of the genes involved in stress resistance are house-keeping genes and so their presence does not necessarily reveal

anything noteworthy for the candidate strain. In fact, many of these genes are involved in central cellular activities so it is unusual that they would not be part of the organism's genome. Therefore, Papadimitriou and colleagues[77] recommended that the decision is based on the enzymatic activity of the proteins in question and not simply the presence of specific genes. This is a valid suggestion that researchers could adopt. In fact, this approach is illustrated by Koskenniemi et al.[124], who identified a BSH enzyme in addition to bile stress-related genes and proteins in bile-stressed *Lb. rhamnosus* GG.

Identification of probiotic strains using molecular markers is a promising approach that will facilitate rapid selection of the desired organisms. Omics technology offers, for the first time, the proper tools for understanding the *in vivo* behavior of probiotics unlike in simulated settings where pure or a handful of microbial strains are used.[77] As omics technologies become cheaper, genome sequencing of candidate strains will become a routine practice.[77] In fact, *in silico* assessment of candidate probiotic strains will also be possible with an incorporation of novel functional transcriptomics and proteomics data into databases.

As earlier stated, up-regulation of protective or repair proteins and stress genes does not necessarily indicate tolerance to a stress. This is indeed insufficient in classifying an organism as acid or bile-salt tolerant. It is, therefore, suggested that for the time being, omics assays be combined with other tests, such as GIT simulation, tube/wells assays and animal models, among others, for more definitive results.

Bile salts hydrolase (BSH)/deconjugation activity

As earlier mentioned, hydrolysis of bile salts produces deconjugated bile salts that have a stronger antimicrobial effect on pathogens than the conjugated forms.[126] Some probiotic organisms reduce cholesterol through bile salt deconjugation.[127] In turn, new bile salts have to be synthesized from the cholesterol in the body.[87] As a result, the hypocholesteromic effect caused by the probiotics could prevent hypercholesterolemia. For instance, Nguyen et al.[128] reported a significant cholesterol reduction of 7% in mice fed on *Lb. plantarum* PH04. Furthermore, Abd El-Gawad et al.[129] observed a 50.3% reduction in total serum cholesterol in rats fed on buffalo milk yogurts containing *B. longum* Bb-46. The ability of probiotic strains to hydrolyze bile salts also helps increase their survival and persistence in the intestine.[49]

BSH activity is assessed by spotting[87] or streaking[28] freshly grown culture on respective agar plates supplemented with bile salts and incubated at appropriate conditions depending on the candidate strains. Mohanty and Ray[87] used 0.5% (w/v) sodium cholate and 0.37 g/L $CaCl_2$ while Banwo et al.[28] used 0.5% (w/v) sodium glycodeoxycholate and 0.5% (w/v) taurodeoxycholate. The concentration used in these studies (0.5% w/v) is reflective of human hepatic duct bile, which is 0.7% bile salts (75). BSH activity is indicated by precipitation of deconjugated bile salts around the colonies.[87] Surono[130] used a similar method, but instead of spotting the culture directly on the agar, sterile paper discs were impregnated with the test cultures and placed on the agar plates. Strains were categorized based on the diameter of the precipitation zone. BSH activity can be categorized as low BSH activity (up to 10 mm), medium BSH activity (11–15 mm) and high BSH activity (>16 mm).[29] Borah et al.[27] used Bile Esculin agar plates containing 4% ox bile. Hydrolysis of the bile esculine produced dark brown to black coloration in the agar. This assay is very simple, reliable and can be used for multiple strains. However, although bile salt hydrolase activity is among the selection criteria

for probiotics, hydrolysis of these salts could be potentially harmful to the human host.[49] Therefore, it is not quite certain if BSH activity is a desirable probiotic trait. Large amounts of deconjugated bile salts are feared to have detrimental effects on the human host. For example, impaired lipid digestion caused by deconjugated bile salts, which are not very efficient in emulsification of dietary lipids.[131] Additionally, it is proposed that subsequent modification of deconjugated bile salts may cause DNA damage, promote colon cancer, impair colonic mucosal function and form gallstones.[132–135] Fortunately, the bacteria genera most likely to be used as probiotics (*Lactobacilli* and *Bifidobacteria*) are incapable of dehydroxylating deconjugated bile salts.[136,137] Therefore, the breakdown products of BSH activity will mainly be precipitated and eliminated from the body through stool.[49]

Evaluation of probiotic activity or benefit

Probiotic organisms confer different health benefits to their host (see section 3). These benefits are conferred while the organisms are in the ileum. Therefore, a candidate strain should have the ability to attach onto the epithelium lining of the ileum. This is discussed in this section together with the evaluation of antimicrobial activity against pathogens (based on; Joint FAO/WHO Working Group.[73]). Other probiotic activities that can be assessed are summarized in Table 2.

Attachment to epithelium lining

In addition to tolerance of the harsh gastrointestinal conditions, probiotic organisms should be able to attach onto the epithelium of the ileum before they start to provide their health benefits.[138] This ability prevents the peristaltic movements of the digestive tract from removing the organisms before they confer their benefits.[139] Furthermore, adhesion to, and colonization of, the mucosal surfaces inhibits pathogens through competition for binding sites and nutrients.[140,141] Probiotics are short-term colonizers of the GIT because they replicate in the intestine to a small extent.[142] They should, therefore, preferably be consumed on a daily basis to maintain their numbers and effectiveness.[143] Attachment of microorganisms to the epithelial lining can be evaluated by using human cell lines, animal models and genomics.

Human cell lines. Evaluation of the attachment capabilities of strains is often done using *ex vivo* models because they are the closest to *in vivo* studies in humans. Human colon cell lines, Cancer coli-2 (Caco-2) and HT29, which are continuous cells of heterogeneous human epithelial colorectal adenocarcinoma, are commonly used.[144,145] Other cell lines used in probiotic characterization include the HeLa cell line[146], Madin-Darby Canine Kidney (MDCK)[147], HEp-2 and T84 cell lines.[148] When cultured under specific conditions, the cell lines differentiate and polarize making their phenotype, morphology and functionality resemble the enterocytes of mature ileum[149] The HT-29 cells differentiate less than the Caco-2 cells.[150] Although the HT-29 line has a typical morphology of epithelial cells, it does not form a typical brush border as is the case with the Caco-2 cells. For that reason, the Caco-2 cells are often used to study transepithelial transport and to some extent epithelial barrier function[151] while HT-29 cells are mainly used to study intestinal bacterial adherence.[152] HT-29 MTX cells (a mutant of HT-29 cells) have better barrier properties than Caco-2 cells.[153] This is because a large portion of the HT-29 cell

line is comprised of goblet-like cells, which produce large amounts of mucin.[154] Mucin has been suggested to aid in the modulation of the adhesion of live organisms to the epithelial surface.[155] Therefore, to compensate for the lacking mucus production, a co-culture model based on Caco-2 and the HT-29-MTX cell line has been developed.[151]

Given the heterogeneity of Caco-2 cells, cultivation conditions can be manipulated to select for the growth of specific cell subpopulations resulting in a model with properties different from the original cell line.[151] As a result, researchers have optimized the Caco-2 cell culture protocol to produce a highly polarized monolayer of cells that display various characteristics of the intestinal enterocytes.[151]

The obvious disadvantage with the use of Caco-2 and HT-29 cell lines is that they are cancer cells and may react differently from normal intestinal epithelial cells.[157] In addition, the tissues rarely survive for long outside the human body, thus restricting the study design. The lack of circulation can also significantly alter the tissue responses to the processes induced during the study.[156] The study of strains in the absence of additional microbiota that would mimic the typical gut microbiome is another limitation because it is known that fierce competition occurs for adhesion sites among various microbes *in vivo*.[77] Nonetheless, intestinal cell models are popular for their simplicity and reproducibility.[151] They make it possible to study molecular mechanisms that would otherwise be difficult to achieve *in vivo*.[151] Strains that adhere with high efficiency to human cell lines *ex vivo* usually behave similarly *in vivo*.[77] Much as intestinal cell lines are a powerful tool for studying the adhesion properties of organisms to the intestinal epithelium, caution should be taken when extrapolating results from *ex vivo* models to *in vivo* cases.

Animal models. Animal intestines have also successfully been used to study the adhesion of organisms to the epithelium of the ileum. For instance, Kos et al.[157] held Landras pig ileum at 4°C for 30 min in phosphate buffered saline solution to loosen the surface mucus and then washed it using the same solution. This was followed by incubation of the tissue in a 10^9/mL cell suspension of bacteria at 37°C for 30 min. Samples were fixed in formalin and dehydrated in increasing concentrations of ethanol and then embedded in paraffin. Sections were stained and examined microscopically for cell adhesion. Abbasiliasi et al.[158] used a slightly similar assay but they instead used goat ileum. In both studies, the bacteria were able to adhere to the ileum. However, removing mucin, a natural component of the epithelium, in this test may slightly change the characteristics of the epithelium, thus affecting adhesion. In another study, Saxami et al.[159] fed Wistar rats on milk containing *Lactobacillus casei* ATCC 393 for 7 days. Thereafter, the intestinal content and tissue were subjected to microbiological and molecular analysis. The use of Wistar rats is a much closer approximation to natural gastric transit as it also includes a food matrix, moreover within an animal system.

Genomics. Genomics is also employed in probiotic studies to identify proteins associated with adhesion of organisms onto the intestinal epithelium. For instance, genomic analysis indicates that the presence of FbpA protein may be responsible for adhesion of a probiotic organism to the matrix of epithelial cells.[160] In addition, through genomics, gene clusters responsible for biosynthesis of pili were reported in genomes of probiotic *Lactobacilli*[161] and *Bifidobacteria*.[162] However, the presence of such genes may not be conclusive for the ability of an organism to attach onto the ileum. Therefore, we suggest that genomics is

combined with other traditional techniques such as human cell line and animal models for more definitive results.

Hydrophobicity

According to García et al.[163], hydrophobicity is a measure of the relative tendency of a substance to prefer a nonaqueous rather than an aqueous environment. In an aqueous environment, there is a tendency of water molecules to exclude nonpolar groups. This results in the nonpolar groups associating only among themselves. Hydrophobicity enables the initial contact between an organism and the host cells through the interaction of nonpolar groups on the cell surface.[164] It is desired that a probiotic organism has a high cell surface hydrophobicity. High surface hydrophobicity indicates the capability to attach to the epithelial lining of the intestine and resist the peristaltic movement of food in the intestines.[165]

Cell hydrophobicity is determined by measuring the microbes' adhesion to hydrocarbons. A washed overnight grown culture pellet is suspended in a buffering agent such as phosphate buffered saline and absorbance (A_0) read at 600 nm. Thereafter, a nonpolar phase, for example, toluene[27], dichloromethane[99], n-hexadecane[88] or xylene[140], is added to the cell suspension to form a two-phase system. The suspension is vortexed for 2 min and incubated at room temperature[27,99,139] or 37°C[88] for 15–30 min to allow for phase separation and attachment of the microorganisms in the nonpolar phase. The aqueous phase is carefully separated and its absorbance read at 600 nm (A_1) to determine the portion of microbial cells that did not attach to the nonpolar phase. The percentage of cell surface hydrophobicity (measure of attachment of microbial cells in the nonpolar phase) is then calculated as $(1 - A_1/A_0) \times 100$.

Auto-aggregation and co-aggregation

Bacterial auto-aggregation refers to aggregation of genetically identical bacteria cells while co-aggregation refers to aggregation of genetically distinct bacterial cells.[166] The ability of microorganisms to auto-aggregate correlates with adhesion and is important for colonization and subsequent infection of the GIT.[139] Co-aggregation of probiotics with pathogens may form a barrier that prevents colonization by pathogens in the GIT. Co-aggregation with other probiotics could increase their colonization potential if they are to be used as probiotic co-cultures.[157]

To determine auto-aggregation, Kos et al.[157] vortexed 4 mL of about 8 log cfu/mL of cell suspension for 10 sec and incubated it at room temperature for 5 h. At hourly intervals during the 5 h incubation period, 0.1 mL of the upper suspension was transferred to another tube containing 3.9 mL of phosphate buffered saline and absorbance measured at 600 nm. Percentage auto-aggregation was calculated as $1 - (A_1/A_0) \times 100$, where A_1 is the absorbance at time t = 1, 2, 3, 4 or 5 h, and A_0 is the absorbance at t = 0.

To determine co-aggregation, Kos et al.[157] vortexed equal volumes (2 mL) each of LAB and other microorganism (LAB or pathogen) together for 10 sec. Control tubes of 4 mL of each bacterial suspension were used. The suspensions were incubated at room temperature for 5 h. Absorbance of the mixed and single cultures was measured at 600 nm in a similar way as in the auto-aggregation assay. Percentage co-aggregation was calculated as $[\{(A_x + A_y)/2 - (A_{x+y})\}/(A_x + A_y)/2] \times 100$, where x and y represent each of the two strains in the control tubes and (x + y) represent the co-culture.

Antimicrobial activity

Microorganisms exert their probiotic effect through inhibition of pathogens using organic acids, hydrogen peroxide, alcohols and bacteriocins, among others.[167] The antimicrobial action can also be by displacement or competition with the pathogen for nutrients or attachment sites on the epithelium.[140] Inhibition of mucosal attachment is either by enhancing the function of the mucosal barrier or direct immunological effect on the mucosal immune system. Failure of pathogens to attach onto the epithelium prevents their establishment and toxin production.[141] As pointed out earlier, *in vitro* production of antimicrobial compounds does not necessarily guarantee *in vivo* application. It is uncertain if the microbe under *in vivo* conditions will produce the antimicrobial compound or even in sufficient amounts to cause significant pathogen inhibition.[77] In addition, the effect of antimicrobial compounds produced by probiotic organisms on host tissue is almost never evaluated. Often antimicrobial assays presume that the antimicrobial compound(s) produced by the test strain are harmless to the host's cells[77] and so their safety is not evaluated. However, there are some studies that evaluated the safety of antimicrobial compounds produced by probiotics. For instance, Das and Goyal[168] characterized plantaricin DM5, a bacteriocin produced by *Lb. plantarum* DM5. The cytotoxicity analysis of the bacteriocin on human embryonic kidney 293 (HEK 293) and human cervical cancer (HeLa) cell lines revealed that it was nontoxic and biocompatible. Todorov et al.[169] tested bacteriocin ST8Sh produced by *Lb. plantarum* ST8Sh on the human hepatocellular carcinoma cell line (Huh7.5) for its cytotoxicity profile. It was reported that 25 μg/mL of the bacteriocin was highly toxic, reducing cell viability by about 80%, but at a lower level (5 μg/mL) no cell toxicity was observed. Therefore, in our opinion, for safety reasons, evaluating the potential negative effects of antimicrobial compounds produced by candidate probiotic strains on host cells should be considered.

Agar diffusion assays

Well diffusion. This method is very popular in the study of antimicrobial activity of bacteria.[27,28,99,102,105,158] The microorganism is grown in a broth overnight and centrifuged to obtain the supernatant. Wells of known diameter, usually 4 mm, are made in agar that is pre-inoculated with a pathogen. The wells are filled with the supernatant and plates incubated at appropriate conditions. Clear zones of inhibition around the wells are then measured. Alternatively, the pathogen is spread on the agar surface and the candidate strain spotted on the agar surface. The plates are incubated and inhibition zones measured. These diffusion assays are simple and reliable; nevertheless, like other assays that involve agar diffusion, they are limited by the agar type and density.

Spot-on-the-lawn. The candidate strain is spot inoculated and grown in soft agar and overlaid with another soft agar containing the pathogen and incubated. Inhibition, indicated by a zone of clearing around the producer colony is measured.[170] To confirm the production of a bacteriocin-like compound, Byaruhanga et al.[170] filled holes made in the agar next to the LAB with proteases and incubated the plates at 30°C for 3 h before overlaying. Plates were then checked for proteolysis of the inhibitory substance. However, in this study, no strains were found to produce bacteriocins.

Alternatively, a much simpler assay can be used to determine bacteriocin action. It involves heating the supernatant obtained from the broth of the culture grown overnight at 100°C for 15 min. The antimicrobial activity of the supernatant is then evaluated using

the well-diffusion technique. Though very simple, this method could be misleading in instances where the bacteriocin is heat stable. Quite similarly, to investigate antimicrobial activity due to the action of organic acids, Surono[130] neutralized the cell-free supernatant with 1N NaOH and sterilized it by membrane filtration. The neutralized supernatant was then tested against selected pathogens using the agar spot test method.

Evaluating the safety of probiotics

The metabolic activity of an organism in its host should not result in the production of harmful substances. The safety of potential probiotic organisms can be evaluated by determining if the organism converts food components or biological secretions into harmful secondary metabolites such as biogenic amines, phenols and indole, among others. For evaluation of microbial safety, the Joint FAO/WHO Working Group[73] recommends, at a minimum, the following tests: antibiotic susceptibility, toxin production, hemolysis and assessment of side effects in human studies. Other tests include platelet activation/aggregation, gelatinase activity, DNase activity, lecithinase activity and mucin degradation among others.

Antibiotic susceptibility

It is important that probiotic organisms are not inhibited by antibiotics, because strains with intrinsic antibiotic resistance could in fact be useful for restoring gut microbiota after antibiotic therapy.[171] Antibiotic-resistant probiotics could also play a role in preventing antibiotic-induced diarrhea. Therefore, antibiotic resistance is a point of concern only if there is a risk of transferring antibiotic resistance genes to pathogens.[171] However, speculations about the transfer of antibiotic resistance genes from probiotic bacteria to gut pathogens[172] are yet to be scientifically validated. There is still a need for thorough investigations into the possibility of transfer of antibiotic resistance genes to food and the gut environment.[173]

Antibiotics either kill the microbial cell or simply stop microbial replication without necessarily causing cell death.[174] Tetracyclines, macrolides and sulphonamides are examples of classes of antibiotics that have a bacteriostatic effect while beta-lactams, fluoroquinolones and aminoglycosides are bactericidal.[174] Tests that can be used to study antibiotic susceptibility include the agar disc diffusion test and the Minimum Inhibitory Concentration (MIC) test.

Minimum inhibitory concentration (MIC) method. The MIC method determines the lowest concentration of antibiotic (µg/mL or mg/L) that inhibits the visible growth of an organism.[175] The MIC assay can be performed by tube or macrobroth dilution, antimicrobial gradient methods (E-test), broth microdilution and agar methods.

Tube or macrobroth MIC dilution. This is the simplest method, and it involves inoculating the bacteria in broth tubes containing an antibiotic of varying concentrations. The culture is incubated at specific conditions as per the candidate strain and growth determined using optical density.[176] This method is associated with challenges such as being tedious, risk of making errors during reagent preparations, numerous reagents and large experimental space required.[177] This method is, however, relatively cheap and simple to execute.

Antimicrobial gradient method (E-test). The E-test is a modification of the tube dilution MIC method. A strip with a decreasing concentration of specific antibiotic along its length is used.[175] Pre-poured agar plates are swabbed with the bacteria and the strip placed on the agar surface and incubated. The diameters of inhibition zones around the strip are measured. The MIC value is the concentration along the strip at the intersection of the strip and the growth inhibition ellipse.[175] The E-test can also be used to study antibiotic interactions by placing different strips in succession following a set time interval. The antibiotic interaction is detected by a decrease in the MIC of the combination compared to the value of the most active antibiotic tested alone.[178] Despite the high costs of the strips (about USD 5 per strip and a retail pack contains a minimum of 10 strips), the E-test is far less laborious and a simpler alternative to the MIC tube dilution method.[175] There is also a good correlation between MIC values obtained using the E-test and broth dilution MIC method.[179]

Broth microdilution. This involves dispensing a known volume (usually 50 μL) of varying concentrations of an antibiotic into microtitre plates. The culture (50–100 μL) is then added to the wells and incubated at appropriate conditions depending on the candidate strain. To determine the MICs, Mayhofer et al.[180] read the lowest concentration of antibiotic at which visible microbial growth was inhibited. Alternatively, colorimetric methods based on dyes such as resazurin and tetrazolium salt dyes can be used to determine the MIC endpoint.[181,182] The broth microdilution test is highly reproducible and correlates strongly with E-test results.[180] According to Luber et al.[183], it is also a fast, technically simple, low-cost method for MIC determination and can also be automated. Broth microdilution allows for evaluation of multiple antibiotics at once. However, unlike the E-test and other agar-based methods, isolated resistant colonies, which are an important observation, might not be detectable using a broth-based assay.[184] These isolated colonies are believed to be subpopulations of the original strain that exhibit increased resistance to the given antibiotic.[177]

Agar method. Ocaña et al.[185] determined the MIC of *Lactobacilli* using the agar assay. In this assay the antibiotic was added to molten agar (45°C) to final concentrations of 1–1000 μg/mL and then inoculated with the culture and allowed to solidify. The agar plates were incubated at 37°C for 48 h and MIC determined by macroscopic observation.

Agar disc diffusion. The agar disc diffusion method is perhaps the commonest used in antibiotic susceptibility studies. Pre-poured agar plates are swabbed with the bacteria or cell suspensions mixed in soft agar and allowed to solidify. Disks impregnated with a given concentration of antibiotic are then placed on the agar surface. The plates are incubated at appropriate conditions, and the diameters of clear zones around the disks are measured.[186,187] Unfortunately, since the clear zones do not necessarily mean cell death, this method cannot differentiate between bactericidal and bacteriostatic antibiotics.[175] It, however, gives an indication of microbial inhibition. In addition, the method does not determine MIC because it is not possible to quantify the amount of antibiotic that diffused into the agar.[175] Only an approximation of the MIC can be made using data of the clear zones and previous algorithms.[188] MIC is defined as the lowest concentration of antibiotic (mg/mL) that completely inhibits the growth of an organism.[189] It guides the drug exposure necessary to ensure maximal efficacy in the event of infection; generally, drug concentrations need to be 4 to 5 times the MIC to ensure the antibiotic is effective.[190]

Antibiotic diffusion in this method is highly dependent on the type, thickness, pH and moisture content of the agar used as well as the inoculum concentration and incubation conditions.[191,192] It is vital to minimize the interference of these factors with the results. Nonetheless, the method is preferred for its simplicity, reliability, low cost, ability to test numerous strains against a number of antibiotics and ease of interpreting results.[175] For interpreting such results, publications, including Clinical and Laboratory Standard Institute (CLSI)[193], European Committee on Antimicrobial Susceptibility Testing[194] and Charteris et al.[187], can be consulted. The limitation of these publications is that some of the interpretations are developed based on the antibiotic susceptibility of clinical pathogens rather than probiotic organisms. Others are based on a single genus of lactic acid bacteria, notably *Lactobacillus*. There are also slight variations in the breakpoints among these publications for the similar antibiotics and microorganisms. There is no literature for susceptibility breakpoints for probiotic lactic acid bacteria using some antibiotics such as novobiocin, levofloxacin, streptomycin, cephalexin and amoxicillin, among others. Nonetheless, these publications offer a good option for antibiotic susceptibility categorization of candidate probiotic strains.

Results from the disc diffusion assays categorize a strain as susceptible, intermediate or resistant to a specific antibiotic. According to the CLSI[193], 'susceptible' means '*I*solates are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used.' 'Intermediate' 'Includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels, and for which response rates may be lower than for susceptible isolates. The intermediate category implies clinical efficacy in body sites where the drugs are physiologically concentrated (e.g. quinolones and β-lactams in urine) or when a higher than normal dosage of a drug can be used (e.g. β-lactams). This category also includes a buffer zone, which should prevent small, uncontrolled, technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.' In other words, this category implies that an infection due to the isolate may only be appropriately treated by that particular drug in body sites where the drug is physiologically concentrated for instance in urine (for quinolones) but not in blood and tissues. Alternatively, infection by isolate can only be treated when a higher than normal dose of the drug is used. 'Resistant' *means* 'Isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules, and/or that demonstrate MICs or zone diameters that fall in the range where specific microbial resistance mechanisms (e.g., β-lactamases) are likely, and clinical efficacy of the agent against the isolate has not been reliably shown in treatment studies.' In other words, resistant isolates unlike 'susceptible isolates' and 'intermediate isolates', are not inhibited at all by the usually achievable levels of drug as per normal dosage schedules. The known normal drug dosage for such infection maybe in the range where microbial resistance mechanisms are likely and drug efficacy has not been reliable in previous cases.

According to Borriello et al.[195], for probiotic therapy, the organism to be used should be susceptible to at least two major antibiotics. The major antibiotics are based on either their chemical or molecular structures; that is, β-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulphonamides, glycopeptides and oxazolidinones, among others.[196,197] Alternatively, they are based on their mode of action, which is inhibition of cell wall synthesis, cell membrane function, nucleic acid function, protein synthesis and

metabolic pathways.[187,198] Although MIC values are essential in probiotic screening, we suggest that these should be restricted to genera with a known history of pathogenicity such as *Streptococcus, Enterococcus, Bacillus* and *Escherichia*, among others. MIC values may be of little or no significance for organisms with a GRAS status. Antibiotic susceptibility testing for GRAS strains may only be useful for evaluating survival during and after antibiotic treatment. Thus, the disc diffusion assay, in which the antibiotic concentration of the disc is equivalent to therapeutic dosages used in the unlikely event of probiotic infection, is sufficient. Therefore, using MIC values may be unnecessary when evaluating GRAS strains more so when financial resources are limited.

Other methods. Other antibiotic susceptibility assays include Thin-Layer Chromatography (TLC) – bioautography, time-kill test (time-kill curve), ATP bioluminescence assay and diffusion methods; namely, agar plug diffusion method, cross streak method and the poisoned food method.[176] These methods are often used to study clinical pathogens. To our knowledge, none of them have been used in probiotic screening tests. These methods give rapid results and offer a better understanding of an antibiotic's impact on cell viability. Unfortunately, they are not widely used because of the need for specified equipment and further evaluation for reproducibility and standardization.[176]

Production of biogenic amines

Biogenic amines are organic compounds produced in foods due to the decarboxylase activity of microorganisms; mainly lactic acid bacteria[200], as a defense mechanism against acidic environments.[201] Generally, foods high in biogenic amines are fermented foods or foods exposed to microbial contamination during processing and storage.[202] Examples of biogenic amines include histamine, tyramine, phenylethylamine, putrescine, agmatine and cadaverine[203] and the precursor amino acids include arginine, ornithine, histidine, phenylalanine and tyrosine. Ingestion of foods containing high levels of biogenic amines is associated with vasoactivity, psychoactivity and some biogenic amines are potential precursors of nitrosamines when nitrosatable agents are present in the food.[204] It is thus important that a probiotic organism does not produce biogenic amines in foods.

To determine biogenic amine production, strains are sub-cultured twice in decarboxylating broth containing 2% L-histidine-monohydrochloride, L-tyrosine disodium salt, or L-ornithine monohydrochloride.[85] The broth is then supplemented with 1 μL of a corresponding amino acid precursor such as tyrosine and 1 mg/L pyridoxal 5 phosphate and incubated at 37°C for 72 h.[85] The test strain (1 μL) is then streaked[85] or spotted[203] on different decarboxylase medium plates and incubated under appropriate conditions as per the requirements of the strain. A positive reaction is indicated by a purple halo, except for decarboxylation media containing tyrosine, where a positive reaction is indicated by a clear halo surrounding the colonies.[203]

Research by Priyadarshani and Rakshit[205] showed production of biogenic amines, histamine and tyramine in the decarboxylate broth by *Lb. casei* TISTR 389 and *Lb. delbrueckii* ssp *bulgaricus* TISTR 895. However, it was not observed for *Lb. acidophilus, Lb. lactis* ssp *lactis, L. lactis* ssp *lactis* and *Lb. plantarum*. This confirmed that biogenic amine production is strain dependent and not related to the species. Careful screening of strains for amino acid decarboxylase activity is crucial.

Hemolytic test

It is important that the organism does not cause lysis of red blood cells (RBC) in the body. For some pathogens, hemolysin plays a vital role in their virulence.[85] Hemolysin production is analyzed using Columbia agar plates supplemented with sterile sheep blood.[86] The hemolysis is interpreted based on the extent of hydrolysis of RBCs. Partial hydrolysis is indicated by a green zone (α- hemolysis), complete hydrolysis by a clear zone (β-hemolysis) and no reaction (γ-hemolysis) around the colony.[28] Borah et al.[27] used a similar approach but instead used Blood agar base No.2 plates containing defibrinated sheep blood.

There are currently no published reports of hemolytic activity by *Lactobacilli* and *Bifidobacteria*. Some of the strains known for hemolysis belong to the genera *Bacilli, Streptococci* and *Enterococci*[206–208], so strains belonging to these genera must be evaluated for hemolysis.

Platelet activation/aggregation

This is a crucial factor in the pathogenesis of infective endocarditis.[209] Bacteria can leak into the bloodstream due to gastrointestinal injuries, surgery or dental injuries and promote septicemia by aggregation of platelets.[210] The leakage of bacteria in the circulatory system can contribute to platelet-fibrin clot formation on the endothelial surface.[210] As a result, the physiological ability of platelets to bind fibrinogen causes platelet aggregation and thus, vascular thrombosis or infective endocarditis.

The platelet activation/aggregation assay involves drawing blood from individuals. Individuals should be nonsmokers and should not have taken alcohol in at least the previous two days prior to drawing of blood. The blood is centrifuged to obtain platelet-rich plasma, which is immediately mixed with a buffer (such as Tyrode's buffer, HEPES buffered saline, etc.) and platelet aggregators or candidate bacterial cells.[211] Azizpour et al.[210] used Thrombin Receptor Activator Peptide-6 (TRAP-6) while Korpela et al.[212] used Adenosine Diphosphate (ADP) and adrenaline as triggers for platelet aggregation. On the other hand, Zhou et al.[211] used ADP, Epinephrine (EPN) and *Streptococcus sanguis* as positive controls and Prostaglandin (PGE_1) as a negative control. Samples are incubated at appropriate conditions, and a fixative agent such as formaldehyde added to halt the reactions. A mixture of monoclonal antibodies is then added in each of the tubes and incubated. The monoclonal antibodies are used as biomarkers for activated platelets.[210] The platelets are washed with a buffer and subjected to flow cytometry analysis. The percentage of platelet-specific antibodies and Mean Fluorescence Intensity (MFI) index are utilized to assess the level of platelet activation or aggregation. This technique is accurate but inter-individual variation in the degree of augmentation of platelet aggregation has been reported.[212] Therefore, to counter this, it is recommended that whole blood samples are also analyzed to reduce artificial effects due to individual variation.[211]

The effect of several LAB strains or species including *Lb. acidophilus* NCCB 47025, *Lb.* ssp *plantarum* NCCB 46042, *Lb. rhamnosus* NCCB 98073, *Lb. rhamnosus* GG ATCC 53103, *Lb. rhamnosus* HN001 and *B. lactis* HN019 on platelet activation/aggregation have been evaluated.[210–212] To date, none of the organisms belonging to the LAB above has been implicated in platelet aggregation. Platelet aggregation has mainly been reported in genera *Aerococcus* and *Staphylococcus*[213,214] and in LAB belonging to the *Enterococcus, Streptococcus* genera.[211,213,215] It would appear, therefore, that evaluation of platelet

aggregation is very crucial in LAB or non-LAB organisms where platelet aggregation has been reported previously or when no information on the platelet aggregation potential of the species in question exists.

DNase activity

DNases are extracellular endonucleases that cleave the phosphodiester bond in the backbone of DNA releasing free nucleotides and phosphate and thus, disrupting cell functionality.[216] The DNase test is mainly used for identification of *Staphylococcus aureus* but it can also be done for other organisms. DNase activity is determined by streaking the organism on DNase agar and incubating the plates at conditions appropriate for the test strain.[87] Clear and pink zones around the colonies indicate DNase activity.[217]

Serratia, Staphylococcus, Campylobacter and *Moraxella*[218] are DNase producing genera. To date, there are no reports of DNase activity among lactic acid bacteria and *Bifidobacteria*. Therefore, the DNase activity test may not be entirely relevant when dealing with lactic acid bacteria.

Gelatinase activity

The mucoid lining of the GIT constitutes the target across which several substances are exchanged in the body and gelatinase activity would disrupt it. This would interfere with the normal functioning of the lining and facilitate infections.[219] It is therefore important that candidate probiotic strains do not express gelatinase activity.

In the gelatinase activity assay, the organism is spotted onto agar containing gelatin and incubated at appropriate conditions depending on the candidate strain. Thereafter, the plates are flooded with saturated ammonium sulfate solution and observed for clear zones surrounding the colonies.[219] Formation of a clear zone is positive for gelatinase activity. In another approach, Botes et al.[34] incubated the inoculated plates for 24 h at 37°C and followed this by holding the plates at 4°C for 5 h. Colonies with surrounding opaque zones were regarded gelatinase positive.

Gelatinase activity is usually associated with the following genera; *Bacillus, Clostridia, Proteus, Pseudomonas, Serratia* and *Staphylococcus*.[220] A review of literature reveals *Enterococcus* as the only lactic acid bacteria genera with gelatinase activity.[221,222] Therefore, we suggest gelatinase activity screening for only the genera known for gelatinase production.

Lecithinase test

Bacterial lecithinases are a safety concern because of their role in pathogenicity. Lecithinase can cause hemolysis[223] and membrane disruption.[224] To determine lecithinase activity, Bhat et al.[224] spotted an overnight grown culture on nutrient agar supplemented with 8% egg yolk emulsion and incubated at 30°C. A halo or precipitation zone around the colonies is considered positive for lecithinase activity. *Bacillus cereus* can be used as a positive control.

Some of the species of the genera *Bacillus, Clostridia, Staphylococcus, Pseudomonas* and *Listeria*[226–231] are known for lecithinase activity. There are so far no reports of lecithinase positive *Lactobacilli* and *Bifidobacteria*.

Mucin degradation

The ability of an organism to excessively degrade mucin may allow it to escape the trapping action of the mucin layer. This, in turn, allows it and other commensal or pathogenic organisms access into the bloodstream through the mucosal surface. Degradation of mucin also exposes the underlying epithelial cells to corrosion by stomach acid and pepsin. Some *Lactobacilli* and *Bifidobacteria* strains may have the ability to degrade mucus.[232]

To investigate microbial mucin degradation, Abe et al.[232] and Delgado et al.[113] used partially purified pig gastric mucin. The mucin was added to an agarified medium with and without glucose as the sole carbon source. Diluted suspensions of the strains were spotted into the agar plates and incubated. The plates were then stained with 0.1% amido black in 3.5 mol/L acetic acid for 30 min and then washed with 1.2 mol/L acetic acid. A mucin lysis zone indicated by a discolored halo around the colony was considered a positive result. For the positive control, a fecal slurry grown overnight in Brain Heart Infusion broth was used while *Lb. casei* was used as a negative control. In addition to the above-mentioned agar plate method, Abe et al.[232] investigated this trait by growing the candidate strains in a basal medium. In some media, the carbon source was mucin or glucose, or a combination of mucin and glucose. After incubation, bacterial growth was assessed by measuring the pH and absorbance at 600 nm of the medium. Thereafter, the basal medium of the strain was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine any changes in the composition of the mucin after incubation. Autoclaved feces were used as a negative control. Ruas-Madiedo et al.[233] used gel permeation chromatography to investigate mucin degradation of candidate strains. The decrease in the highest molecular mass peak was considered an indicator of mucin degradation.

The agar technique is a simple and quick way to evaluate microbial mucin degradation but like all other agar assays, this method could be limited by the type of agar used. Measurement of absorbance is a rapid method of determining microbial growth but metabolites often interfere with the results. SDS-PAGE and gel permeation chromatography are accurate techniques, they not only indicate mucin degradation, but they also make it possible to identify the actual changes in the mucin composition by means of bands or peaks of the degradation compounds.

Future prospects for probiotic research

The future for the screening of probiotics should focus on addressing the limitations of both the current assays and the guidelines for selecting and declaring probiotic microorganisms. To facilitate probiotics research, it is important that cheaper, simpler and faster screening techniques whose results are accurate, reliable and reproducible are available/developed. Guidelines could also be reviewed to include considerations for: (1) establishment of dosage for probiotic efficacy, (2) determination of allergenicity of probiotic organisms, (3) specification of simple, accurate and precise *in vitro* and *in vivo* screening assays and (4) evaluation of antimicrobial compounds produced by probiotics for negative effects on host cells. If taken into consideration, these recommendations have the capacity to revolutionize probiotic screening protocols.

Conclusion

Guidelines for screening and declaration of probiotics exist and can be used to validate candidate strains to avoid flooding the market with 'probiotic products' whose authenticity cannot be scientifically justified. This paper reviewed the different methods used in the characterization of probiotic strains. Though there are major limitations associated with some of the traditional *in vitro* and *in vivo* assays, they still remain very useful in probiotic studies. More sophisticated approaches pertaining to probiotics research such as omics technology are underway and will be perfected with time. It is these new technological advancements that will address the limitations of the traditional assays.

Acknowledgments

Thanks to Dr. Ueli von Ah for permission to use the illustration on stomach-intestinal passage simulation using a bioreactor. Thanks to Dr. Gurudayal Singh Toteja as well for permission to use the illustration on guidelines for evaluation of candidate probiotics strains developed by the Indian Council of Medical Research (ICMR) and Department of Biotechnology (DBT). We acknowledge the Food Technology and Business Incubation Center (FTBIC), Makerere University, Kampala for its contribution to this work.

References

[1] Grand View Research. **2016**. Probiotics Market Analysis by Application (Probiotic Functional Foods and Beverages, Probiotic Dietary Supplements, Animal Feed Probiotics), by End Use (Human Probiotics, Animal Probiotics) and Segment Forecasts to 2020. http://www.grandviewresearch.com/industry-analysis/probioticsmarket (accessed Nov 5, 2016).

[2] Markets and Markets. **2017**. Probiotics Market Worth 64.02 Billion USD by 2022. https://www.marketsandmarkets.com/PressReleases/probiotics.asp (accessed Dec 20, 2017).

[3] Mukisa, I. M. **2016**. Probiotics: What Is Africa Doing? https://www.researchgate.net/publication/310319829_Probiotics_what_is_Africa_doing (accessed Dec 20, 2017).

[4] Global Industry Analysts Incorporated. **2016**. Probiotics – A Global Strategic Business Report. www.strategyr.com/pressMCP-1084.asp (accessed Jul 22, 2017).

[5] Kechagia, M.; Basoulis, D.; Konstantopoulou, S.; Dimitriadi, D.; Gyftopoulou, K.; Skarmoutsou, N.; Fakiri, E. M. Health Benefits of Probiotics: A Review. *ISRN Nutrition*, 2013, *2013*, 1–7. DOI: 10.5402/2013/481651

[6] Ziemer, C. J.; Gibson, G. R. An Overview of Probiotics, Prebiotics and Synbiotics in the Functional Food Concept: Perspectives and Future Strategies. *Int. Dairy J.* **1998**, *8*(5–6), 473–479. DOI: 10.1016/S0958-6946(98)000.

[7] Granato, D.; Branco, G. F.; Nazzaro, F.; Cruz, A. G.; Faria, J. A. Functional Foods and Nondairy Probiotic Food Development: Trends, Concepts, and Products. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*(3), 292–302. DOI: 10.1111/j.1541-4337.2010.00110.x.

[8] Metchnikoff, E.;. *Essais Optimistes. The Prolongation of Life Optimistic Studies*; Heinemann: London UK, 1907.

[9] Tissier, H.;. *Recherchers sur la flora intestinale normale et pathologique du nourisson*; University of Paris: France, 1900.

[10] Travers, M.-A.; Sow, C.; Zirah, S.; Deregnaucourt, C.; Chaouch, S.; Queiroz, R. M. L.; Charneau, S.; Allain, T.; Florent, I.; Grellier, P. Deconjugated Bile Salts Produced by Extracellular Bile Salt Hydrolase-Like Activities from the Probiotic *Lactobacillus Johnsonii* La1inhibit *Giardia Duodenalis in Vitro* Growth. *Front. Microbiol.* **2016**, *7*(1453), 1–16. DOI: 10.3389/fmicb.2016.01453.

[11] Kumar, M.; Kumar, A.; Nagpal, R.; Mohania, D.; Behare, P.; Verma, V.; Kumar, P.; Pddae, D.; Aggarwal, P. K.; Henry, C. J.;, et al. Cancer-Preventing Attributes of Probiotics: An Update. *Int. J. Sci. Nutr.*.**2010**, *61*(5), 473–496. DOI: 10.3109/09637480903455971.
[12] Yan, F.; Polk, D. B. Probiotics as Functional Food in the Treatment of Diarrhea. *Curr. Opin. Clin. Nutr. Metabol. Care*. **2006**, *9*, 717–721. DOI: 10.1097/01.mco.0000247477.02650.51.
[13] Ogawa, M.; Shimizu, K.; Nomoto, K.; Takahashi, M.; Watanuki, M.; Tanaka, R.; Hamabata, T.; Yamasaki, S.; Takeda, Y. Protective Effect of *Lactobacillus Casei* Strain Shirota on Shiga Toxin-Producing *Escherichia Coli* O157: H7infection in Infant Rabbits. *Infect. Immunol.* **2001**, *69*, 1101–1108. DOI: 10.1128/IAI.69.2.1101-1108.2001.
[14] Lin, M. Y.; Chen, T. W. Reduction of Cholesterol by *Lactobacillus Acidophilus* in Culture Broth. *J. Food Drug Anal.* **2000**, *8*(2), 97–102.
[15] Fuller, R.;. Probiotics for Farm Animals. In *Probiotics a Critical Review*; Tannock, G. W., Ed.; Horizon Scientific, Wymondham: UK, 1999; pp 15–22.
[16] Fuller, R.;. Probiotics in Man and Animals. *J. Appl. Bacteriol.* **1989**, *66*(5), 365–378. DOI: 10.1111/j.1365-2672.1989.tb05105.x.
[17] FAO/WHO. **2006**. Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. http://www.fao.org (accessed Dec 28, 2016).
[18] Cunha, A. F.; Acurcio, L. B.; Assis, B. S.; Oliveira, D. L. S.; Leite, M. O.; Cerqueira, M. M. O. P.; Souza, M. R. *In Vitro* Probiotic Potential of Lactobacillus Spp. Isolated from Fermented Milks. *Arq. Bras. Med. Vet. Zootec.* **2013**, *65*(6), 1876–1882. DOI: 10.1590/S0102-09352013000600040.
[19] Minelli, E. B.; Benini, A. Relationship between Number of Bacteria and Their Probiotic Effects. *Microb. Ecol. Health Dis.* **2008**, *20*(4), 180–183. DOI: 10.1080/08910600802408095.
[20] Sanders, M. E.;. Probiotics: Definition, Sources, Selection, and Uses. *Clin. Infect. Dis.* **2008**, *46*(2), S58– S61. DOI: 10.1086/523341.
[21] Whorwell, P. J.; Altringer, L.; Morel, J.; Bond, Y.; Charbonneau, D.; O'Mahony, L.; Kiely, B.; Shanahan, F.; Quigley, E. M. Efficacy of an Encapsulated Probiotic *Bifidobacterium Infantis* 35624 in Women with Irritable Bowel Syndrome. *Am. J. Gastroenterol.* **2006**, *101*, 1581–1590. DOI: 10.1111/j.1572-0241.2006.00734.x.
[22] Gionchetti, P.; Rizzello, F.; Helwig, U. M.; Venturi, A.; Lammers, K. M.; Brigidi, P.; Vitali, B.; Poggioli, G.; Miglioli, M.; Campieri, M. Prophylaxis of Pouchitis Onset with Probiotic Therapy: A Double-Blind, Placebo-Controlled Trial. *Gastroenterol.* **2003**, *124*(5), 1202–1209. DOI: 10.1016/S0016-5085(03)00171-9.
[23] Shornikova, A. V.; Casas, I. A.; Mykkanen, H.; Salo, E.; Vesikari, T. Bacteriotherapy with *Lactobacillus Reuteri* in Rotavirus Gastroenteritis. *Pediatr. Infect. Dis. J.* **1997**, *16*, 1103–1107. DOI: 10.1097/00006454-199712000-00002.
[24] Market Data Forecast. **2016**. Middle-East and Africa Probiotics Market by Bacteria. www.marketdataforecast.com/market-reports/middle-east-and-africa-probiotics-market-776/ (accessed Jul 23, 2017).
[25] Global Probiotics Market **2017-2021**. 2017. https://www.technavio.com/report/global-probiotics-market (accessed Jun 30, 2017).
[26] Lei, V.; Jakobsen, M. Microbiological Characterization and Probiotic Potential of *Koko* and *Koko* Sour Water, African Spontaneously Fermented Millet Porridge and Drink. *J. Appl. Microbiol.* **2004**, *96*, 384–397. DOI: 10.1046/j.1365-2672.2004.02162.x.
[27] Borah, D.; Gogoi, O.; Adhikari, C.; Kakoti, B. B. Isolation and Characterization of the New Indigenous *Staphylococcus* Sp. DBOCP06 as a Probiotic Bacterium from Traditionally Fermented Fish and Meat Products of Assam State. *Egypt. J. Basic Appl. Sci.* **2016**, *3*, 232–240. DOI: 10.1016/j.ejbas.2016.06.001.
[28] Banwo, K.; Sanni, A.; Tan, H. Technological Properties and Probiotic Potential of *Enterococcus Faecum* Strains Isolated from Cow Milk. *J. Appl. Microbiol.* **2013**, *114*, 229–241. DOI: 10.1111/jam.12031.
[29] Mathara, J. M.; Schillinger, U.; Guigas, C.; Franz, C.; Kutima, P. M.; Mbugua, S. K.; Shin, H.-K.; Holzapfel, W. H. Functional Characteristics of *Lactobacillus* Spp. From Traditional

Maasai Fermented Milk Products in Kenya. *Int. J. Food Microbiol.* **2008**, *126*, 57–64. DOI: 10.1007/s00284-007-9084-6.
[30] Saxelin, M.; Tynkkynen, S.; Mattila-Sandholm, T.; de Vos, W. M. Probiotic and Other Functional Microbes: From Markets to Mechanisms. *Curr. Opin. Biotechnol.* **2005**, *16*, 204–211. DOI: 10.1016/j.copbio.2005.02.003.
[31] Rabah, H.; Rosa Do Carmo, L. R.; Jan., G. Dairy Propionibacteria: Versatile Probiotics. *Microorg.* **2017**, *5*(2). DOI: 10.3390/microorganisms5020024.
[32] Jayanthi, N.; Ratan Sudha, M. *Bacillus Clausii* - the Probiotic of Choice in the Treatment of Diarrhea. *J. Yoga Phys. Ther.* **2015**, *5*(4), 1–4. DOI: 10.4172/2157-7595.1000211.
[33] Otte, J.-M.; Mahjurian-Namari, R.; Brand, S.; Werner, I.; Schmidt, W. E.; Schmitz, F. Probiotics Regulate the Expression of COX-2 in Intestinal Epithelial Cells. *Nutr. Cancer.* **2009**, *61*(1), 103–113. DOI: 10.1080/01635580802372625.
[34] Botes, M.; van Reenen, C. A.; Dicks, L. M. T. Evaluation of *Enterococcus Mundtii* ST4SA and *Lactobacillus Plantarum* 423 as Probiotics by Using a Gastro-Intestinal Model Infant Milk Formulations as Substrate. *Int. J. Food Microbiol.* **2008**, *128*, 362–370. DOI: 10.1016/j.ijfoodmicro.2008.09.016.
[35] Nangia, T.; Setia, V.; Kochhar, G. K.; Kaur, K.; Bansal, R.; Sharma, R. Probiotics: Review of Literature. *J. Period. Med. Clin. Pract.* **2014**, *1*, 144–151.
[36] Hong, H. A.; Huang, J. H.; Khanej, R.; Hiep, L. V.; Urdaci, M. C.; Cutting, S. M. The Safety of *Bacillus Subtilis* and *Bacillus Indicus* as Food Probiotics. *J. Appl. Microbiol.* **2008**, *105*, 510–520. DOI: 10.1111/j.1365-2672.2008.03773.x.
[37] Cutting, S. M.;. *Bacillus* probiotics. *Food Microbiol.* **2011**, *28*(2), 214–220. DOI: 10.1016/j.fm.2010.03.007.
[38] Soccol, C. R.; Vandenberghe, L. P.; Spier, M. R.; Medeiros, A. B. P.; Yamaguishi, C. T.; Lindner, J. D. D.; Pandey, A.; Thomaz-Soccol, V. The Potential of Probiotics: A Review. *Food Technol. Biotechnol.* **2010**, *48*(4), 413–434.
[39] Araújo, T. F.; Ferreira, C. L. F. The Genus *Enterococcus* as Probiotic: Safety Concerns. *Braz. Arch. Biol. Tech.* **2013**, *56*(3). DOI: 10.1590/S1516-89132013000300014.
[40] Goh, H. M. S.; Yong, M. H. A.; Chong, K. K. L.; Kline, K. A. Model Systems for the Study of Enterococcal Colonization and Infection. *Virulence.* **2016**, *8*, 1525–1562. DOI: 10.1080/21505594.2017.1279766.
[41] Nami, Y.; Hagshenas, B.; Hagshenas, M.; Khosroushahi, A. Antimicrobial Activity and Presence of Virulence Factors and Bacteriocin Structural Genes in *Enterococcus Faecium* CM33 Isolated from Ewe Colostrum. *Front. Microbiol.* 2015, *6*(782). DOI: 10.3389/fmicb.2015.00782.
[42] Lodemann, U.; Strahlendorf, J.; Schierack, P.; Klingspor, S.; Aschenbach, J. R.; Martens, H. Effects of the Probiotic *Enterococcus Faecium* and Pathogenic *Escherichia Coli* Strains in a Pig and Human Epithelial Intestinal Cell Model. *Scientifica (Cairo).* **2015**, *2015*, 1–10. DOI: 10.1155/2015/235184.
[43] Al Atya, A. K.; Drider-Hadiouche, K.; Ravallec, R.; Silvain, A.; Vachee, A.; Drider, D. Probiotic Potential of *Enterococcus Faecalis* Strains Isolated from Meconium. *Front. Microbiol.* **2015**, *6*(227). DOI: 10.3389/fmicb.2015.00227.
[44] Sonnenborn, U.; Schulze, J. The Non-Pathogenic *Escherichia Coli* Strain Nissle 1917 – Features of a Versatile Probiotic. *Microb. Ecol. Health Dis.* **2009**, *21*, 122–158. DOI: 10.3109/08910600903444267.
[45] Schultz, M.;. Clinical Use of *E. Coli* Nissle 1917 in Inflammatory Bowel Disease. *Inflamm. Bowel Dis.* **2008**, *14*, 1012–1018. DOI: 10.1002/ibd.20377.
[46] Clinical-Trials. **2017**. Study to Determine the Effectiveness of the Probiotic *E. Coli* Strain M17 in Treating Irritable Bowel Syndrome (IBS). https://clinicaltrials.gov/ct2/show/NCT00194922 (accessed Jul 25, 2017).
[47] Kotowska, M.; Albrecht, P.; Szajewska, H. *Saccharomyces Boulardii* in the Prevention of Antibiotic-Associated Diarrhea in Children: Randomized Double-Blind Placebo-Controlled Trial. *Aliment. Pharm.Ther.* **2005**, *21*(5), 583–590. DOI: 10.1111/j.1365-2036.2005.02356.x.

[48] Biviano, I.; Rossi, S.; Paicentino, D.; Alvino, V.; Corazziari, E. S.; Badiali, D.; Gastroenterology, A. Effect of *Bifidobacterium Longum* Bb536 Plus Lactoferrin in the Treatment of Irritable Bowel Syndrome. A Double Blind Clinical Trial. *Adv. Res. Gastroenterol. Hepatol.* **2017**, *6*(4), 1–4. DOI: 10.19080/argh.2017.06.555691.
[49] Begley, M.; Hill, C.; Gahan, C. G. M. Bile Salt Hydrolase Activity in Probiotics. *Appl. Environ. Microbiol.* **2006**, *72*(3), 1729–1738. DOI: 10.1128/AEM.72.3.1729-1738.2006.
[50] Assimos, D. G.;. Probiotic Therapy for Hyperoxaluria. *Rev. Urol.* **2006**, *8*(3), 170–171.
[51] LeBlanc, J. G.; Rutten, G.; Bruinenberg, P.; Sesma, F.; de Giori, G. S.; Smid, E. J. A Novel Dairy Product Fermented with *Propionibacterium Freudenreichii* Improves the Riboflavin Status of Deficient Rats. *Nutr.* **2006**, *22*, 645–651. DOI: 10.1016/j.nut.2006.01.002.
[52] Doron, S.; Snydman, D. R.; Gorbach, S. L. *Lactobacillus* GG: Bacteriology and Clinical Applications. *Gastroenterol. Clin. North Am.* **2005**, *34*, 483–498. DOI: 10.1016/j.gtc.2005.05.011.
[53] LeBlanc, J. G.; Burgess, C.; Sesma, F.; de Giori, G. S.; van Sinderen, D. *Lactococcus Lactis* Is Capable of Improving the Riboflavin Status in Deficient Rats. *Br. J. Nutr.* **2005**, *94*, 262–267.
[54] Reid, G.; Bruce, A. W.; Taylor, M. Instillation of Lactobacillus and Stimulation of Indigenous Organisms to Prevent Recurrence of Urinary Tract Infections. *Microecol. Ther.* **1995**, *23*, 32–45.
[55] Prazdnova, E. V.; Chistyakov, V. A.; Churilov, M. N.; Mazanko, M. S.; Bren, A. B.; Volski, A.; Chikindas, M. L. DNA-protection and Antioxidant Properties of Fermentates from *Bacillus Amyloliquefaciens* B-1895 and *Bacillus Subtilis* KATMIRA 1933. *Lett. Appl. Microbiol.* **2015**, *61*(6), 549–554. DOI: 10.1111/lam.12491.
[56] Kort, R.; Westerik, N.; Mariela Serrano, L.; Douillard, F. P.; Gottstein, W.; Mukisa, I. M.; Tuijn, C. J.; Basten, L.; Hafkamp, B.; Meijer, W. C.; Teusink, B.; de Vos, W. M.; Reid, G.; Sybesma, W. A novel consortium of *Lactobacillus rhamnosus and Streptococcus thermophilus* for increased access to functional fermented foods. *Microb. Cell Fact.* **2015**, *14*(1), 1–14. DOI: 10.1186/s12934-015-0370-x.
[57] Drisko, J. A.; Giles, C. K.; Bischoff, B. J. Probiotics in Health Maintenance and Disease Prevention. *Altern. Med. Rev.* **2003**, *8*(2), 143–155.DOI: 10.12938/bifidus.25.39.
[58] Ducrotté, P.; Sawant, P.; Jayanthi, V. Clinical Trial: Lactobacillus Plantarum 299v (DSM 9843) Improves Symptoms of Irritable Bowel Syndrome. World J. Gastroenterol. **2012**, 18 1205 (30),4012–4018. DOI: 10.3748/wjg.v18.i30.4012.
[59] Matsumoto, K.; Takada, T.; Shimizu, K.; Kado, Y.; Kawakami, K.; Makino, I.; Yamaoka, Y.; Hirano, K.; Nishimura, A.; Kajimoto, O.; et al. The Effect of a Probiotic Milk Product Containing Lactobacillus Casei Strain Shirota on the Defecation Frequency and the Intestinal Microflora of Sub-Optimal Health State Volunteers: A Randomized Placebo-Controlled **Cross-Over** Study. *Biosci. Microflora.* 2006, *25*(2), 39–48. DOI: 10.12938/bifidus.25.39.
[60] Symonds, E. L.; O'Mahony, C.; Lapthorne, S.; O'Mahony, D.; Sharry, J. M.; O'Mahony, L.; Shanahan, F. *Bifidobacterium Infantis* 35624 Protects against Salmonella-Induced Reductions in Digestive Enzyme Activity in Mice by Attenuation of the Host Inflammatory Response. Clin. Transl. Gastroenterol. **2012**, *3*(5), e15. DOI: 10.1038/ctg.2012.9.
[61] Bruno, F.; Frigerio, G. A New Therapeutic Alternative for The Treatment Of Enteritis-Controlled Double-blind Test with The Strain Sf 68. *Schweiz. Rundsch. Med. Prax.* **1981**, *70* (39), 1717–1720.
[62] Kruis, W.; Fric, P.; Pokrotnieks, J.; Lukás, M.; Fixa, B.; Kascak, M.; Kamm, M. A.; Weismueller, J.; Beglinger, C.Stolte, M.; et al. Maintaining Remission of Ulcerative Colitis with the Probiotic Escherichia Coli Nissle **1917** Is as Effective as with Standard Mesalazine. *Gut*, **2004**, *53*, 1617–1623. DOI: 10.1136/gut.2003.037747.
[63] Wojtyniak, K.; Horvath, A.; Dziechciarz, P.; Szajewska, H. *Lactobacillus Casei Rhamnosus* Lcr35 in the Management of Functional Constipation in Children: A Randomized Trial. *J. Pediatr.* **2017**, *184*, 101–105. DOI: 10.1016/j.jpeds.2017.01.068.

[64] Petricevic, L.; Witt, A. The Role of *Lactobacillus Casei Rhamnosus* Lcr35 in Restoring the Normal Vaginal Flora after Antibiotic Treatment of Bacterial Vaginosis. *Gen. Gynaecol.* **2017**, 1369–1374. DOI: 10.1111/j.1471-0528.2008.01882.x.
[65] Martín, R.; Laval, L.; Chain, F.; Miquel, S.; Natividad, J.; Cherbuy, C.; Sokol, H.; Verdu, E. F.; Vlieg, J. H.; Bermudez-Humaran, L. G.; et al. *Bifidobacterium Animalis* Ssp. *Lactis* CNCM-I2494 Restores Gut Barrier Permeability in Chronically Low-Grade Inflamed Mice. *Front. Microbiol.* **2016**, *7*(608), 1–12. DOI: 10.3389/fmicb.2016.00608.
[66] Sgouras, D. N.; Panayotopoulou, E. G.; Martinez-Gonzalez, B.; Petraki, K.; Spyros Michopoulos, S.; Mentis, A. Lactobacillus Johnsonii La1 Attenuates Helicobacter Pylori –Associated Gastritis and Reduces Levels of Pro Inflammatory Chemokines in C57BL/6 Mice. Clin. Diagn. Lab. Immunol. **2005**, 12(12),1378–1386. DOI: 10.1128/CDLI.12.12.1378-1386.2005.
[67] Dietrich, C. G.; Kottmann, T.; Alavi, M. Commercially Available Probiotic Drinks Containing Lactobacillus Casei DN-114001 Reduce Antibiotic-Associated Diarrhea. *World J. Gastroenterol.* **2014**, *20*(42), 15837–15844. DOI: 10.3748/wjg.v20.i42.15837.
[68] Bruns, R.; Raedsch, R. Therapy of Traveller's Diarrhea. *Med. Welt.* **1995**, *46*, 591–596.
[69] Ringel-Kulka, T.; Palsson, O. S.; Maier, D.; Carroll, I.; Galanko, J. A.; Leyer, G.; Ringel, Y. Probiotic Bacteria Lactobacillus Acidophilus NCFM and Bifidobacterium Lactis Bi-07 versus 1245Placebo for the Symptoms of Bloating in Patients with Functional Bowel Disorders: A Double-Blind Study. J. Gastroenterol. **2011**, 45(6),518–525. DOI: 10.1097/MCG.0b013e31820ca4d6 .
[70] Ardeypharm. **2018**. Discover the German Group of Companies. www.ardeypharm.com/Probiotical-pharmaceuticals.html (accessed Jan 26, 2018).
[71] Jäger, R.; Purpura, M.; Farmer, S.; Cash, H. A.; Keller, D. Probiotic *Bacillus Coagulans* GBI-30, 6086 Improves Protein Absorption and Utilization. *Probiotics Antimicrob. Proteins.* **2017**. DOI: 10.1007/s12602-017-9354-y.
[72] Medellin-Peña, M. J.; Griffiths, M. W. Effect of Molecules Secreted by *Lactobacillus Acidophilus* Strain La-5 on *Escherichia Coli* O157: H7colonization. *Appl. Environ. Microbiol.* **2009**, *75*(4), 1165–1172. DOI: 10.1128/AEM.01651-08.
[73] Joint FAO/WHO Working Group. **2002**. Report on Drafting Guidelines for the Evaluation of Probiotics in Food: Guidelines for the Evaluation of Probiotics in Food. *London.* http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf (accessed Jun 30, 2017).
[74] Mahasneh, A. M.; Abbas, M. M. Probiotics and Traditional Fermented Foods: The Eternal Connection (Mini-Review). *Jordan J. Biol. Sci.* **2010**, *3*(4), 133–140.
[75] Ganguly, N. K.; Bhattacharya, S. K.; Sesikeran, B.; Nair, G. B.; Ramakrishna, B. S.; Sachdev, H. P. S.; Batish, V. K.; Kanagasabapathy, A. S.; Muthuswamy, V.; Kathuria, S. C. ICMR-DBT Guidelines for Evaluation of Probiotics in Food. *Indian J. Med. Res.* **2011**, *134*(1), 22–25.
[76] Ramos, C. L.; Thorsen, L.; Schwan, R. F.; Jespersen, L. Strain-Specific Probiotics Properties of *Lactobacillus Fermentum, Lactobacillus Plantarum and Lactobacillus Brevis* Isolates from Brazilian Food Products. *Food Microbiol.* **2013**, *36*(1), 22–29. DOI: 10.1016/j.fm.2013.03.010.
[77] Papadimitriou, K.; Zoumpopoulou, G.; Foligne, B.; Alexandraki, V.; Kazou, M.; Pot, B.; Tsakalidou, E. Discovering Probiotic Microorganisms: *In Vitro, in Vivo*, Genetic and Omics Approaches. *Front. Microbiol.* **2015**, *6*(58), 1–28. DOI: 10.3389/fmicb.2015.00058.
[78] Adams, M. R.; Marteau, P. On the Safety of Lactic Acid Bacteria from Food. *Int. J. Food Microbiol.* **1995**, *27*, 263–264. DOI: 10.1016/0168-1605(95)00067-T.
[79] Marteau, P.;. Safety Aspects of Probiotic Products. *Näringsforskning.* **2001**, *45*(1), 22–24. DOI: 10.3402/fnr.v45i0.1785.
[80] Snydman, D. R.;. The Safety of Probiotics. *Clin. Infect.* **2008**, *46*, S104– 111. DOI: 10.1086/523331.
[81] Kurokawa, K.; Itoh, T.; Kuwahara, T.; Oshima, K.; Toh, H.; Toyoda, A.; Takama, H.; Morita, H.; Sharma, V. K.; Srivastva, T. P.;, et al. Comparative Metagenomics Revealed Commonly Enriched Gene Sets in Human Gut Microbiomes. *DNA Res.* **2007**, *14*, 169–181. DOI: 10.1093/dnares/dsm018.
[82] Khaenhammer, T. R.; Kullen, M. J. Selection and Design of Probiotics. *Int. J. Food Microbiol.* **1999**, *50*(1–2), 45–57. DOI: 10.1016/S0168-1605(99)00076-8.

[83] Kõll, P.; Mändar, R.; Marcotte, H.; Leibur, E.; Mikelsaar, M.; Hammarstöm, L. Characterization of Oral Lactobacilli as Potential Probiotics for Oral Health. Oral Microbiol. Immunol. **2008**, 23(2),139–147. DOI: 10.1111/j.1399-302X.2007.00402.x.
[84] Rahman, S. M. K.; Jalil, A.; Rahman, S. M. M.; Hossain, K. M. A Study on Probiotic Properties of Isolated and Identified Bacteria from Regional Yoghurts. *Int. J. Biosci.* **2015**, *13107*(4), 139–149. DOI: 10.12692/ijb/7.4.139-149.
[85] Belicová, A.; Mikulášová, M.; Dušinský, R. Probiotic Potential and Safety Properties of Lactobacillus Plantarum from Slovak Bryndza Cheese. Biomed. Res. Int. **2013**, 760298,1 – 8. DOI: 10.1155/2013/760298.
[86] Leite, A. M. O.; Miguel, M. A. L.; Peixoto, R. S.; Ruas-Madiedo, P.; Paschoalin, V. M. F.; Mayo, B.; Delgado, S. Probiotic Potential of Selected Lactic Acid Bacteria Strains from Brazilian Kefir Grains. J. Dairy Sci. **2015**, 98, 3622–3632. DOI: 10.3168/jds.2014-9265.
[87] Yadav, R.; Puniya, A. K.; Shukia, P. Probiotic Properties of Lactobacillus Plantarum RYPR1 from an Indigenous Fermented Beverage Raabadi. Front. Microbiol. **2016**, 7(1683),1–9. DOI: 10.3389/fmicb.2016.01683.
[88] Shukla, R.; Goyal, I. I. A. Leuconostoc Mesenteroids NRRL B-1149 as Probiotic and Its Dextran with Anticancer Properties. J. Biosci. Biotechnol. **2014**, 3(1),79–87.
[89] Osmanagaoglu, O.; Kiran, F.; Yagci, F. C.; Gursel, I. Immunomodulatory Function and in Vivo Properties of Pediococcus Pentosaceus OZF, a Promising Probiotic Strain. Ann. Microbiol. **2012**, 63(4),1311–1318. DOI: 10.1007/s13213-012-0590-9.
[90] Ritter, P.; Kohler, C.; von Ah, U. Evaluation of the Passage of Lactobacillus Gasseri K7 and Bifidobacteria from the Stomach to Intestines Using a Single Reactor Model. BMC Microbiol. **2009**, 9(87),1–9. DOI: 10.1186/1471-2180-9-87
[91] Hou, R. C.; Lin, M. Y.; Wang, M. M.; Tzen, J. T. Increase of Viability of Entrapped Cells of *Lactobacillus Delbrueckii* Spp. *Bulgaricus* in Artificial Sesame Oil Emulsions. *J. Dairy Sci.* **2003**, *86*, 424–428. DOI: 10.3168/jds.s0022-0302(03)73620-0.
[92] Begley, M.; Gahan, C. G.; Hill, C. The Interaction between Bacteria and Bile. *FEMS Microbiol. Rev.* **2005**, *29*, 625–651. DOI: 10.1016/j.femsre.2004.09.003.
[93] Flahaut, S.; Hartke, A.; Giard, J. C.; Benachour, A.; Boutibonnes, P.; Auffray, A. Relationship between Stress Response Towards Bile Salts, Acid and Heat Treatment in *Enterococcus Faecalis*. *FEMS Microbiol. Lett.* **1996**, *138*, 49–54. DOI: 10.1111/j.1574-6968.1996.tb08133.x.
[94] Shimakawa, Y.; Matsubara, S.; Yuki, N.; Ikeda, M.; Ishikawa, F. Evaluation of *Bifidobacterium Breve* Yakult- Fermented Soymilk as a Probiotic Food. *Int. J. Food Microbiol.* **2003**, *81*, 131–136.
[95] Okoli, A. S.; Raftery, M. J.; Mendz, G. L. Effects of Human and Porcine Bile on the Proteome of *Helicobacter Hepaticus*. *Proteome Sci.* **2012**, *10*(27), 1–16. DOI: 10.1186/1477-5956-10-27.
[96] Grill, J. P.; Cayuela, C.; Antoine, J. M.; Schneider, F. Isolation and Characterization of a *Lactobacillus Amylovorus* Mutant Depleted in Conjugated Bile Salt Hydrolase Activity: Relation between Activity and Bile Salt Resistance. *J. Appl. Microbiol.* **2000**, *89*, 553–563. DOI: 10.1046/j.1365-2672.2000.01147.x.
[97] Legrand-Defretin, V.; Juste, C.; Henry, R.; Corring, T. Ion-Pair High-Performance Liquid Chromatography of Bile Salt Conjugates: Application to Pig Bile. *Lipids*. **1991**, *26*, 578–583. DOI: 10.1007/BF02536421.
[98] Barrett, K.; Brooks, H.; Boitano, S.; Barman, S. **2012**. Ganong's Review of Medical Physiology. https://emergencypedia.files.wordpress.com/2013/04/ganong-pdf.pdf (accessed Dec 24, 2017).
[99] Jose, N. M.; Bunt, C. R.; Hussain, M. A. Comparison of Microbiological and Probiotic Characteristics of *Lactobacilli* Isolates from Dairy Food Products and Animal Rumen Contents. *Microorg.* **2015**, *3*, 198–212. DOI: 10.3390/microorganisms3020198.
[100] Goldin, B. R.; Gorbach, S. L. Probiotics for Humans. In *Probiotics, the Scientific Basis*; Fuller, R., Ed.; Chapman and Hall: London, 1992; pp 355–376.
[101] Rohrig, B.; **2012**. 24 Hours: Your Food on the Move. https://www.acs.org/content/dam/acsorg/education/resources/highschool/chemmatters/videos/chemmatters-feb2012-digestion.pdf (accessed Feb 3, 2018).

[102] Hassanzadazar, H.; Ehsani, A.; Mardani, K.; Hesari, J. Investigation of Antibacterial, Acid and Bile Tolerance Properties of Lactobacilli Isolated from Koozeh Cheese. *Vet. Res. Forum*. **2012**, *3*(3), 181–185.

[103] Xanthopoulos, V.; Litopoulou-Tzanetaki, E.; Tzanetakis, N. Characterization of *Lactobacillus* Isolates from Infant Faeces as Dietary Adjuncts. *Food Microbiol.* **2000**, *17*, 205–215. DOI: 10.1006/fmic.1999.0300.

[104] Dunne, C.; O'Mahony, L.; Murphy, L.; Thornton, G.; Morrissey, D.; O'Hlloran, S.; Feeney, M.; Flynn, S.; Fitzgerald, G.; Daly, C.; et al. *In Vitro* Selection Criteria for Probiotic Bacteria of Human Origin: Correlation with *in Vivo* Findings. *Am. J. Clin. Nutr.* **2001**, *73*, 386S–392S. DOI: 10.1093/ajcn/73.2.386s.

[105] Kim, P. I.; Jung, M. Y.; Chang, Y.-H.; Kim, S.; Kim, S.-J.; Park, Y.-H. Probiotic Properties of *Lactobacillus* and *Bifidobacterium* Strains from Porcine Gastrointestinal Tract. *Appl. Microbiol. Biotechnol.* **2007**, *74*, 1103–1111. DOI: 10.1007/s00253-006-0741-7.

[106] Abdulhussein, B. J.; Hussein, Y. F.; Nawar, A. H.; Al-Naggar, R. A. Conversion Rate of Laparoscopic Cholecystectomy to Open Surgery at Al Karamah Teaching Hospital, Iraq. *Surg. Sci.* **2015**, *6*, 221–226. DOI: 10.4236/ss.2015.65034.

[107] Riaz, M. S.; Shaheen, T.; Siddiq, M.; Nadeem, A.; Hussain, A.; Hayyat, F.; Shi, J. In-Vitro Assessment of Probiotic Potential of Lactic Acid Bacteria. *J. Biol. Today's World*. **2015**, *4*(10), 190–198. DOI: 10.15412/J.JBTW.01041001.

[108] Jacobsen, C. N.; Nielsen, V. R.; Hayford, A. E.; Møller, P. F.; Michaelsen, K. F.; Pærregaard, A.; Sandström, B.; Tvede, M.; Jakobsen, M. Screening of Probiotic Activities of Forty-Seven Strains of *Lactobacillus* Spp. By *in Vitro* Techniques and Evaluation of the Colonization Ability of Five Selected Strains in Humans. *Appl. Environ. Microbiol.* **1999**, *65*(11), 4949–4956.

[109] Vinderola, G.; Capellini, B.; Villarreal, F.; Suárez. Usefulness of a set of simple in vitro tests for the screening and identification of probiotic candidate strains for dairy use. *LWT – Food Sci. Tech*. **2008**, *41*, 1678–1688. DOI: 10.1016/j.lwt.2007.10.008.

[110] Todorov, S. D.; Botes, M.; Guigas, C.; Schillinger, U.; Wiid, I.; Wachsman, M. B.; Holzapfel, W. H. Boza, a Natural Source of Probiotic Lactic Acid Bacteria. *J. Appl. Microbiol.* **2008**, *104*, 465–477. DOI: 10.1111/j.1365-2672.2007.03558.x.

[111] Duangjitcharoen, Y.; Kantachote, D.; Ongsakul, M.; Poosaran, N.; Chaiyasut, C. Selection of Probiotic Lactic Acid Bacteria Isolated from Fermented Plant Beverages. *Pak. J. Biol. Sci.* **2008**, *11*(4), 652–655. DOI: 10.3923/pjbs.2008.652.655.

[112] Delgado, S.; O'Sullivan, E.; Fitzgerald, G.; Mayo, B. *In Vitro* Evaluation of the Probiotic Properties of Human Intestine *Bifidobacterium* Species and Selection of New Probiotic Candidates. *J. Appl. Microbiol.*. **2008**, *104*, 1119–1127. DOI: 10.1111/j.1365-2672.2007.03642.x.

[113] Picot, A.; Lacroix, C. Encapsulation of Bifidobacteria in Whey Protein-Based Microcapsules and Survival in Simulated Gastrointestinal Conditions and in Yoghurt. *Int. Dairy J.* **2004**, *14*, 505–515. DOI: 10.1016/j.idairyj.2003.10.008.

[114] Meddah, A. T. T.; Yazourh, A.; Desmet, I.; Risbourg, B.; Verstraete, W.; Romond, M. B. The Regulatory Effects of Whey Retentate from Bifidobacteria Fermented Milk on Microbiota of the Simulator of the Human Intestinal Microbial Ecosystem (SHIME). *J. Appl. Microbiol.* **2001**, *91*(6), 1110–1117. DOI: 10.1046/j.1365-2672.2001.01482.x.

[115] Marteau, P.; Minekus, M.; Havenaar, R.; Veld, J. H. J. Survival of Lactic Acid Bacteria in a Dynamic Model of the Stomach and Small Intestine: Validation and Effects of Bile. *J. Dairy Sci.* **1997**, *80*, 1031–1037. DOI: 10.3168/jds.S0022-0302(97)76027-2.

[116] Drouault, S.; Corthier, G.; Ehrlich, S. D.; Renault, P. Survival, Physiology and Lysis of *Lactococcus Lactis* in the Digestive Tract. *Appl. Environ. Microbiol.* **1999**, *65*(11), 4881–4886.

[117] Sumeri, I.; Adamberg, S.; Uusna, R.; Sarand, I.; Paalme, T. Survival of Cheese Bacteria in a Gastrointestinal Tract Simulator. *Int. Dairy J.* **2012**, *25*(1), 36–41. DOI: 10.1016/j.idairyj.2011.12.016.

[118] De Boever, P.; Verstraete, W. Bile Salt Deconjugation by *Lactobacillus Plantarum* 80 and Its Implication for Bacterial Toxicity. *J. Appl. Microbiol.* **1999**, *87*, 345–352. DOI: 10.1046/j.1365-2672.1999.00019.x.

[119] Duangjitcharoen, Y.; Kantachote, D.; Ongsakul, M.; Poosaran, N.; Chaiyasut, C. Potential Use of Probiotic *Lactobacillus Plantarum* SS2 Isolated from a Fermented Plant Beverage: Safety Assessment and Persistence in the Murine Gastrointestinal Tract. *World J. Microbiol. Biotechnol.* **2009**, *25*(2), 315–321. DOI: 10.1007/s11274-008-9894-0.
[120] Horgan, R. P.; Kenny, L. C. SAC Review 'Omics' Technologies: Genomics, Transcriptomics, Proteomics and Metabolomics. *Obstet. Gynaecol.* **2011**, *13*, 189–195. DOI: 10.1576/toag.13.3.189.27672.
[121] Hamon, E.; Horvatovich, P.; Marchioni, E.; Aoudé-Werner, D.; Ennahar, S. Investigation of Potential Markers of Acid Resistance in *Lactobacillus Plantarum* by Comparative Proteomics. *J. Appl. Microbiol.* **2014**, *116*, 134–144. DOI: 10.1111/jam.12339.
[122] Jin, J.; Zhang, B.; Guo, H.; Cui, J.; Jiang, L.; Song, S.; Sun, M.; Ren, F. Mechanism Analysis of Acid Tolerance Response of *Bifidobacterium Longum* Subsp. *Longum* BBMN 68 by Gene Expression Profile Using RNA-sequencing. *PLoS ONE*. **2012**, *7*, e50777. DOI: 10.1371/journal.pone.0050777.
[123] An, H.; Douillard, F. P.; Wang, G.; Zhai, Z.; Yang, J.; Song, S.; Cui, J.; Ren, F.; Luo, Y.; Zhang, B.;, et al. Integrated Transcriptomics and Proteomic Analysis of the Bile Stress Response in a Centenarian - Originated Probiotic *Bifidobacterium Longum* BBMN68. *Mol. Cell Proteomics* **2014**, *13*, 2558–2572. DOI: 10.1074/mcp.M114.039156.
[124] Koskenniemi, K.; Laakso, K.; Koponen, J.; Kankainen, M.; Greco, D.; Auvinen, P.; Savijoki, K.; Nyman, T. A.; Surakka, A.; Salusjärvi, T.;, et al. Proteomics and Transcriptomics Characterization of Bile Stress Response in Probiotic *Lactobacillus Rhamnosus* GG. *Mol. Cell. Proteomics*.**2011**, *10*(2), 1–18. DOI: 10.1074/mcp.M110.002741.
[125] Hamon, E.; Horvatovich, P.; Bisch, M.; Bringe, F.; Marchioni, E.; Aoudé-Werner, D.; Ennahar, S. Investigation of Biomarkers of Bile Tolerance in *Lactobacillus Casei* Using Comparative Proteomics. *J. Proteome Res.* **2012**, *11*(1), 109–118. DOI: 10.1021/pr200828t.
[126] Bermudez-Brito, M.; Plaza-Díaz, J.; Muñoz-Quezada, S.; Gómez-Lorente, C.; Gil, A. Probiotic Mechanisms of Action. *Ann. Nutr. Metab.* **2012**, *61*, 160–174. DOI: 10.1159/000342079.
[127] Liong, M. T.; Shah, N. P. Acid and Bile Tolerance and Cholesterol Removal Ability of Lactobacilli Strains. *J. Dairy Sci.* **2005**, *88*, 55–66. DOI: 10.3168/jds.s0022-0302(05)72662-x.
[128] Nguyen, T. D. T.; Kang, J. H.; Lee, M. S. Characterization of *Lactobacillus Plantarum* PH04, a Potential Probiotic Bacterium with Cholesterol-Lowering Effects. *Int. J. Food Microbiol.* **2007**, *113*, 358–361. DOI: 10.1016/j.ijfoodmicro.2006.08.015.
[129] Abd El-Gawad, I. A.; El-Sayed, E. M.; Hafez, S. A.; El-Zeini, H. M.; Saleh, F. A. The Hypocholesterolaemic Effect of Milk Yoghurt and Soy-Yoghurt Containing *Bifidobacteria* in Rats Fed on Cholesterol-Enriched Diet. *Int. Dairy J.* **2005**, *15*, 37–44. DOI: 10.1016/j.idairyj.2004.06.001.
[130] Surono, I. S.;. *In Vitro* Probiotic Properties of Indigenous *Dadih* Lactic Acid Bacteria. *Asian-Aust. J. Anim. Sci.* **2003**, *16*(5), 726–731. DOI: 10.1007/s00253-008-1553-8.
[131] De Smet, I.; Van Hoorde, I.; De Saeyer, M.; Vande, W. M.; Verstraete, W. *In Vitro* Study of Bile Salt Hydrolase (*BSH*) Activity of *BSH* Isogenic *Lactobacillus Plantarum* 80 Strains and Estimation of Cholesterol Lowering through Enhanced BSH Activity. *Microb. Ecol. Health Dis.* **1994**, *7*, 315–329. DOI: 10.3109/08910609409141371.
[132] Bernstein, C.; Bernstein, H.; Payne, C. M.; Dvorakova, K.; Garewal, H. Bile Acids as Carcinogens in Human Gastrointestinal Cancers. *Mutat. Res.* **2005**, *589*, 47–65. DOI: 10.1016/j.mrrev.2004.08.001.
[133] Veysey, M. J.; Thomas, L. A.; Mallet, A. I.; Jenkins, P. J.; Besser, G. M.; Wass, J. A.; Murphy, G. M.; Dowling, R. H. Prolonged Large Bowel Transit Increases Serum Deoxycholic Acid: A Risk Factor for Octreotide Induced Gallstones. *Gut.* **1999**, *44*, 675–681.
[134] Pazzi, P.; Puriani, A. C.; Dalla Libera, M.; Guerra, G.; Rici, D.; Gullini, S.; Ottolenghi, C. Bile Salt-Induced Cytotoxicity and Ursodeoxycholate Cytoprotection: In Vitro Study Perfused Rat Hepatocytes. *Eur. J. Gastroenterol. Hepatol.* **1997**, *9*, 703–709. DOI: 10.1097/00042737-199707000-00011.

[135] Kandell, R. L.; Bernstein, C. Bile Salt/Acid Induction of DNA Damage in Bacterial and Mammalian Cells: Implications for Colon Cancer. *Nutr. Cancer*. **1991**, *16*, 227–238. DOI: 10.1080/01635589109514161.

[136] Ahn, Y. T.; Kim, G. B.; Lim, Y. S.; Baek, Y. J.; Kim, Y. U. Deconjugation of Bile Salts by *Lactobacillus Acidophilus* Isolates. *Int. Dairy J.* **2003**, *13*, 303–311. DOI: 10.1016/S0958-6946(02)00174-7.

[137] Takahashi, T.; Morotomi, M. Absence of Cholic Acid 7-Dehydroxylase Activity in the Strains of *Lactobacillus* and *Bifidobacterium*. *J. Dairy Sci.* **1994**, *77*, 3275–3286. DOI: 10.3168/jds.S0022-0302(94)77268-4.

[138] Chou, L. S.; Weimer, B. Isolation and Characterization of Acid- and Bile-Tolerant Isolates from Strains of *Lactobacillus Acidophilus*. *J. Dairy Sci.* **1999**, *82*(1), 23–31. DOI: 10.3168/jds.s0022-0302(99)75204-5.

[139] Tomáška, M.; Drončovský, M.; Klapáčová, L.; Slottová, A.; Kološta, M. Potential Probiotic Properties of *Lactobacilli* Isolated from Goat's Milk. *Potravinarstvo Sci. J. Food Ind.* **2015**, *9*(1), 66–71. DOI: 10.5219/434.

[140] Collado, M. C.; Meriluoto, J.; Salminen, S. Role of Commercial Probiotic Strains against Human Pathogen Adhesion to Intestinal Mucus. *Lett. Appl. Microbiol.* **2007**, *45*, 454–460. DOI: 10.1111/j.1472-765x.2007.02212.x.

[141] Gueimonde, M.; Jalonen, L.; He, F.; Hiramatsu, M.; Salminen, S. Adhesion and Competitive Inhibition and Displacement of Human Enteropathogens by Selected Lactobacilli. *Food Res. Int.* **2006**, *39*, 467–471.

[142] Marco, M. L.; Pavan, S.; Kleerebezem, M. Towards Understanding Molecular Modes of Probiotic Action. *Curr. Opin. Biotechnol.* **2006**, *17*, 204–210. DOI: 10.1016/j.copbio.2006.02.005.

[143] De Champs, C. D.; Maroncle, N.; Balestrino, D.; Rich, C.; Forestier, C. Persistence of Colonization of Intestinal Mucosa by a Probiotic Strain, *Lactobacillus Casei* Subsp. *Rhamnosus* Lcr35, after Oral Consumption. *J. Clin. Microbiol.* **2003**, *41*(3), 1270–1273. DOI: 10.1128/JCM.41.3.1270-1273.2003.

[144] Aissi, E. A.; Lecocq, M.; Brassart, C.; Bouquelet, S. Adhesion of Some Bifidobacteria Strains to Human Enterocyte-Like Cells and Binding to Mucosal Glycoproteins. *Microb. Ecol. Health Dis.* **2001**, *13*, 32–39. DOI: 10.1080/089106001750071681.

[145] Fogh, J.; Trempe, G. New Human Tumor Cell Lines. In *Human Tumor Cells in Vitro*; Fogh, J., Ed.; New Plenum Press: York, 1975; pp 115–141.

[146] Tropcheva, R.; Georgieva, R.; Danova, S. Adhesion Ability of *Lactobacillus Plantarum* AC131. *Biotechnol. Biotechnol. Equip.* **2011**, *25*(1), 121–124. DOI: 10.5504/BBEQ.2011.0123.

[147] Denkova, R.; Strinska, H.; Denkova, Z.; Dobrev, G.; Torodov, D.; Mladenova, K.; Shishkov, S. Study on the Adhesion of *Lactobacillus Plantarum* Strains with Probiotic Properties to MDCK. *J. Fac. Food Eng.* **2014**, *13*(3), 214–217.

[148] Haeri, A.; Khodaii, Z.; Ghaderian, S. M. H.; Panah, A. S. T.; Najar, R. A. Comparison of Adherence Patterns of a Selection of Probiotic Bacteria to Caco-2, HEp-2 and T84 Cell Lines. *Ann. Microbiol.* **2012**, *62*(1), 339–344. DOI: 10.1007/s13213-011-0267-9.

[149] Hidalgo, I. J.; Raub, T. J.; Borchard, R. T. Characterization of the Human Colon Carcinoma Cell Line (Caco-2) as a Model System for Intestinal Epithelial Permeability. *Gastroenterol.* **1989**, *96*(3), 736–749. DOI: 10.1016/0016-5085(89)90897-4.

[150] Merino-Trigo, A.; Rodríguez-Berrocal, F. J.; de Miguel, E.; Páez de la Cadena, M. Activity and Properties of a-L-fucosidase are Dependent on the State of Enterocytic Differentiation of HT-29 Colon Cancer Cells. *Int. J. Biochem. Cell Biol.* **2002**, *34*, 1291–1303. DOI: 10.1016/S1357-2725(02)00067-5.

[151] Lea, T.;. Epithelial Cell Models: General Introduction. In *The Impact of Food Bioactives on Health: In Vitro and Ex Vivo Models*; Verhoeckx, K., Ed.; Springer: Switzerland, 2015; pp 95–109.

[152] Sarem, F.; Sarem-Damerdji, L. O.; Nicolas, J. P. Comparison of the Adherence of Three *Lactobacillus* Strains to Caco-2 and Int-407 Human Intestinal Cell Lines. *Lett. Appl. Microbiol.* **1996**, *22*, 439–442.

[153] Gopal, P. K.; Prasad, J.; Smart, J.; Gill, H. S. *In Vitro* Adherence Properties of *Lactobacillus Rhamnosus* DR20 and *Bifidobacterium Lactis* DR10 Strains and Their Antagonistic Activity against an Enterotoxigenic *Escherichia Coli*. *Int. J. Food Microbiol.* **2001**, *67*, 207–216.
[154] Grajek, W.; Olejnik, A. Epithelial Cell Cultures *in Vitro* as a Model to Study Functional Properties of Food. *Pol. J. Food Nutr. Sci.* **2004**, *13*, 5–24.
[155] Otte, J.-M.; Podolsky, D. K. Functional Modulation of Enterocytes by Gram-Positive and Gram Negative Microorganisms. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2004**, *286*(4), G613– G626. DOI: 10.1152/ajpgi.00341.2003.
[156] Skovdahl, H. K. Differences between the HT29 and HT29 MXT epithelial cell lines. PhD Thesis, Norwegian University of Science and Technology, Norway, **2016**.
[157] Kos, B.; Susković, J.; Vuković, S.; Simpraga, M.; Frece, J.; Matosić, S. Adhesion and Aggregation Ability of Probiotic Strain *Lactobacillus Acidophilus* M92. *J. Appl. Microbiol.* **2003**, *94*, 981–987. DOI: 10.1046/j.1365-2672.2003.01915.x.
[158] Abbasiliasi, S.; Tan, J. S.; Bashokouh, F.; Ibrahim, T. A. T.; Mustafa, S.; Vakhshiteh, F.; Sivasamboo, S.; Ariff, A. B. *In Vitro* Assessment of *Pediococcus Acidilactici* Kp 10 for Its Potential Use in the Food Industry. *BMC Microbiol.* **2017**, *17*(1), 121. DOI: 10.1186/s12866-017-1000-z.
[159] Saxami, G.; Ypsilantis, P.; Sidira, M.; Simopoulos, C.; Kourkoutas, Y.; Galanis, A. Distinct Adhesion of Probiotic Strain *Lactobacillus Casei* ATCC 393 to Rat Intestinal Mucosa. *Anaerobe*. **2012**, *19*, 417–420. DOI: 10.1016/j.anaerobe.2012.04.002.
[160] Azcarate-Peril, M. A.; Altermann, E.; Goh, Y. J.; Tallon, R.; Sanozky-Dawes, R. B.; Pfeiler, E. A.; O'Flaherty, S.; Buck, B. L.; Dobson, A.; Duong, T.;, et al. Analysis of the Genome Sequence of *Lactobacillus Gasseri* ATCC33323 Reveals the Molecular Basis of an Autochthonous Intestinal Organism. *Appl. Environ. Microbiol.* **2008**, *74*, 4610–4625. DOI: 10.1128/AEM.00054-08.
[161] Douillard, F. P.; Ribbera, A.; Järvinen, H. M.; Kant, R.; Pietila, T. E.; Randazzo, C.; Paulin, L.; Laine, P. K.; Caggia, C.; von Ossowski, I.;, et al. Comparative Genomic and Functional Analysis of *Lactobacillus Casei* and *Lactobacillus Rhamnosus* Strains Marked as Probiotics. *Appl. Environ. Microbiol.***2013**, *24*(3), 531–538. DOI: 10.1128/AEM.03467-12.
[162] Turroni, F.; Serafini, F.; Foroni, E.; Duranti, S.; O'connell Motherway, M.; Taver-Niti, V.; Mangifesta, M.; Milani, C.; Viappiani, A.; Roversi, T.;, et al. Role of Sortase-Dependent Pili of *Bifidobacterium Bifidum* PRL2010 in Modulating Bacterium-Host Interactions. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 11151–11156. DOI: 10.1073/pnas.1303897110.
[163] García, M. A.; Marina, M. L.; Ríos, A.; Valcárcel, M. Separation Modes in Capillary Electrophoresis. In *Analysis and Detection by Capillary Electrophoresis*; Marina, M.L., Ríos, A., Valcárcel, M., Eds.; Elsevier: Amsterdam, 2005; pp 31–134.
[164] Shobharani, P.; Agrawal, R. A Potent Probiotic Strain from Cheddar Cheese. *Indian J. Microbiol.* **2011**, *51*(3), 251–258. DOI: 10.1007/s12088-011-0072-y.
[165] Chauviere, G.; Coconnier, M. H.; Kerneis, S.; Darfeuille-Michaud, A. Competitive Exclusion of Diarrheagenic *Escherichia Coli* (ETEC) from Human Enterocyte-Like Caco-2 Cells by Heat Killed. *Lactobacillus FEMS Microbiol. Lett.* **1992**, *70*, 213–217. DOI: 10.1016/0378-1097(92)90700-X.
[166] Grady, C. P. L., Jr.; Daigger, G. T.; Lim, H. C. *Biological Wastewater Treatment*, 2nd ed.; Marcel Dekker: New Yolk, 1999.
[167] Reid, G.; Jass, J.; Sebulsky, M. T.; McCormick, J. K. Potential Uses of Probiotics in Clinical Practice. *Clin Microbiol Rev*. **2003**, *16*, 4658–4672. DOI: 10.1128/CMR.16.4.658-672.2003.
[168] Das, D.; Goyal, A. Characterization of a Noncytotoxic Bacteriocin from Probiotic *Lactobacillus Plantarum* DM5 with Potential as a Food Preservative. *Food Funct.* **2014**, *5*, 2453–2462. DOI: 10.1039/c4fo00481g.
[169] Todorov, S. D.; Perin, L. M.; Carneiro, B. M.; Rahal, P.; Holzapfel, W.; Nero, L. A. Safety of *Lactobacillus Plantarum* ST8Sh and Its Bacteriocin. *Probiotics Antimicrob. Proteins*. **2017**, *9*, 334–344. DOI: 10.1007/s12602-017-9260-3.

[170] Byaruhanga, Y. B.; Bester, B. H.; Watson, T. G. Growth and Survival *of Bacillus Cereus* in Mageu, a Sour Maize Beverage. *World J. Microbiol. Biotechnol.* **1999**, *15*, 329–333. DOI: 10.1023/A:1008967117381.

[171] Gueimonde, M.; Sánchez, B.; de Los Reyes-Gavilán, C. G.; Margolles., A. Antibiotic Resistance in Probiotic Bacteria. *Front. Microbiol.* **2013**, *4*(202), 1–6. DOI: 10.3389/fmicb.2013.00202.

[172] Broaders, E.; Gahan, C. G.; Marchesi, J. R. Mobile Genetic Elements of the Human Gastrointestinal Tract: Potential for Spread of Antibiotic Resistance Genes. *Gut. Microb.* **2013**, *4*, 271–280. DOI: 10.4161/gmic.24627.

[173] Lahtinen, S. J.; Boyle, R. J.; Margolles, A.; Frías, R.; Gueimonde, M. Safety Assessment of Probiotics. In *Prebiotics and Probiotics Science and Technology*; Charalampopoulos, D., Rastall, R.A., Eds.; Springer-Verlag: Berlin, 2009; pp 1193–1225.

[174] Nemeth, J.; Oesch, G.; Kuster, S. P. Bacteriostatic Verses Bacteriocidal Antibiotics for Patients with Serious Bacterial Infections: Systematic Review and Meta-Analysis. *J. Antimicrob. Chemother*. **2015**, *70*(2), 382–395. DOI: 10.1093/jac/dku379.

[175] Balouiri, M.; Sadiki, M.; Ibnsouda, S. K. Methods for *in Vitro* Evaluating Antimicrobial Activity: A Review. *J. Pharm. Anal.* **2016**, *2016*(6), 71–79. DOI: 10.1016/j.jpha.2015.11.005.

[176] Vandenbossche, I.; Vaneechoutte, M.; Vandevenne, M.; De Baere, T.; Verschraegen, G. Susceptibility Testing of Fluconazole by the NCCLS Broth Macrodilution Method, E-Test and Disk Diffusion for Application in the Routine Laboratory. *J. Clin. Microbiol.* **2002**, *40*(3), 918–921. DOI: 10.1128/JCM.40.3.918-921.2002.

[177] Jorgensen, J. H.; Ferraro, M. J. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clin. Infect. Dis.* **2009**, *49*, 1749–1755. DOI: 10.1086/647952.

[178] Denes, È.; Hidri, N. Synergie et antagonism en antibiothèrapie. *Antibiotiques*. **2009**, *11*, 106–115. DOI: 10.1016/j.antib.2009.02.001.

[179] Berghaus, L. J.; Giguère, S.; Guldbech, K.; Warner, E.; Ugorji, U.; Berghaus, R. D. Comparison of *Etest*, Disk Diffusion, and Broth Macrodilution for *in Vitro* Susceptibility Testing of *Rhodococcus Equi. J. Clin. Microbiol.* **2015**, *53*, 314–318. DOI: 10.1128/JCM.02673-14.

[180] Mayhofer, S.; Domig, K. J.; Mair, C.; Zitz, U.; Huys, G.; Kneifel, W. Comparison of Broth Microdilution, Etest, and Agar Disk Diffusion Methods for Antimicrobial Susceptibility Testing of *Lactobacillus Acidophilus* Group Members. *Appl. Environ. Microbiol.* **2008**, *74* (12), 3745–3748. DOI: 10.1128/AEM.02849-07.

[181] Castilho, A. L.; Caleffi-Ferracioli, K. R.; Canezin, P. H.; Dias Sigueira, V. L.; de Lima Scodro, R. B.; Cardoso, R. F. Detection of Drug Susceptibility in Rapidly Growing Mycobacteria by Resazurin Broth Micro-Dilution Assay. *J. Microbiol. Methods*. **2015**, *111*, 119–121. DOI: 10.1016/j.mimet.2015.02.007.

[182] Al-Bakri, A. G.; Afifi, F. U. Evaluation of Antimicrobial Activity of Selected Plant Extracts by Rapid XTT Colorimetry and Bacterial Enumeration. *J. Microbiol. Methods*. **2007**, *2007*(68), 19–25. DOI: 10.1016/j.mimet.2006.05.013.

[183] Luber, P.; Bartelt, E.; Genschow, E.; Wagner, J.; Hahn, H. Comparison of Broth Microdilution, Etest and Agar Dilution Methods for Antibiotic Susceptibility Testing of *Campylobacter Jejuni* and *Campylobacter Coli. J. Clin. Microbiol.* **2003**, *41*(3), 1062–1068. DOI: 10.1128/JCM.41.3.1062-1068.2003.

[184] Danielsen, M.; Wind, A. Susceptibility of *Lactobacillus* spp. *To Antimicrobial Agents. Int. J. Food Microbiol.* **2003**, *82*, 1–11. DOI: 10.1016/S0168-1605(02)00254-4.

[185] Ocaña, V.; Silva, C.; Nader-Macias, M. E. Antibiotic Susceptibility of Potentially Probiotic Vaginal *Lactobacilli. Infect. Dis. Obstet. Gynecol.* **2006**, 1–6. DOI: 10.1155/IDOG/2006/18182.

[186] Kivanç, S. A.; Kivanç, M.; Yiğit, T. Antibiotic Susceptibility, Antibacterial Activity and Characterisation of *Enterococcus Faecum* Strains Isolated from Breast Milk. *Exp. Ther. Med.* **2016**, *12*(3), 1732–1740. DOI: 10.3892/etm.2016.354.

[187] Charteris, W.; Kelly, P.; Morelli, L.; Collins, J. Antibiotic Susceptibility of Potentially Probiotic *Lactobacillus* Species. *J. Food Prot.* **1998**, *61*, 1636–1643. DOI: 10.4315/0362-028X-61.12.1636.
[188] Nijs, A.; Cartuyvels, R.; Mewis, A.; Peeters, V.; Rummens, J. L.; Magerman, K. Comparison and Evaluation of Osiris and Sirscan 2000 Antimicrobial Susceptibility Systems in the Clinical Microbiology Laboratory. *J. Clin. Microbiol.* **2003**, *41*, 3627–3630. DOI: 10.1128/JCM.41.8.3627–3630.2003.
[189] Buller, N.; Thomas, A.; Barton, M. **2014**. Antimicrobial Susceptibility Testing. *Australian and New Zealand Standard Diagnostic Procedures*. http://www.agriculture.gov.au/SiteCollectionDocuments/animal/ahl/ANZSDP-Antimicrobial-susceptibility-testing.pdf (accessed Dec 23, 2017).
[190] Nickson, C. **2017**. Minimum Inhibitory Concentration (MIC). https://lifeinthefastlane.com/ccc/minimum-inhibitory-concentration-mic/(accessed December 23, 2017).
[191] Oxoid. **2017**. Antibiotic Susceptibility Testing Best Practice. http://www.oxoid.com/UK/blue/techsupport/its.asp?itsp=faq&cat=&faq=tsfaq021&c=UK&lang=EN&print=N (accessed Jul 30, 2017).
[192] Huys, G.; D'Haene, K.; Swings, J. Influence of the Culture Medium on Antibiotic Susceptibility Testing of Food Associated Lactic Acid Bacteria with Agar Overlay Disc Diffusion Method. *Lett. Appl. Microbiol.* **2002**, *34*(6), 402–406. DOI: 10.1046/j.1472-765X.2002.01109.x.
[193] Clinical and Laboratory Standard Institute (CLSI). **2013**. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Information Supplement. http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-M100S23-susceptibility-testing-2013-no-protection.pdf (accessed Dec 23, 2017).
[194] European Committee on Antimicrobial Susceptibility Testing. **2013**. Clinical Breakpoints. http://www.eucast.org/clinical_breakpoints/(accessed Dec 23, 2017).
[195] Borriello, S. P.; Hammes, W. P.; Holzapfel, W.; Marteau, P.; Schrezenmeir, J.; Vaara, M.; Valtonen, V. Safety of Probiotics that Contain *Lactobacilli* or *Bifidobacteria*. *Clin. Infect. Dis.* **2003**, *36*, 775–780. DOI: 10.1086/368080.
[196] Etebu, E.; Arikekpar, I. Antibiotics: Classification and Mechanisms of Action with Emphasis on Molecular Perspectives. *Int. J. Appl. Microbiol. Biotechnol. Res.* **2016**, *4*, 90–101.
[197] Adzitey, F.;. Antibiotic Classes and Antibiotic Susceptibility of Bacterial Isolates from Selected Poultry; a Mini Review. *World Vet. J.*. **2015**, *5*(3), 36–41. DOI: 10.5455/wvj.20150853.
[198] Ullah, H.; Ali, S. **2017**. Classification of Anti-Bacterial Agents and Their Functions. https://www.intechopen.com/books/antibacterial-agents/classification-of-anti-bacterial-agents-and-their-functions (accessed Dec 23, 2017).
[199] Lonvaud-Funel, A.;. Biogenic Amines in Wines: Role of Lactic Acid Bacteria. *FEMS Microbiol. Lett.* **2001**, *199*, 9–13. DOI: 10.1111/j.1574-6968.2001.tb10643.x.
[200] Spano, G.; Russo, P.; Lonvaud-Funel, A.; Lucas, P.; Alexandre, H.; Grandvalet, C.; Coton, E.; Coton, M.; Barnavon, L.; Bach, B.;, et al. Biogenic Amines in Fermented Foods. *Eur. J. Clin. Nutr.* **2010**, *64*, 95–100. DOI: 10.1038/ejcn.2010.218.
[201] Shalaby, A. R. Significance of Biogenic Amines to Food Safety and Human Health. *Food Res. Int.* **1996**, *29*, 675–690. DOI: 10.1016/S0963-9969(96)00066-X.
[202] Martín, R.; Olivares, M.; Marín, M. L.; Fernández, L.; Xaus, J.; Rodríguez, J. M. Probiotic Potential of 3 *Lactobacilli* Strains Isolated from Breastmilk. *J. Hum. Lact.* **2005**, *21*(1), 8–17. DOI: 10.1177/0890334404272393.
[203] Bover-Cid, S.; Holzapfel, W. H. Improved Screening Procedure for Biogenic Amine Production by Lactic Acid Bacteria. *Int. J. Food Microbiol.* **1999**, *53*, 33–41.
[204] Priyadarshani, W. M. D.; Rakshit, S. K. Screening Selected Strains of Probiotic Lactic Acid Bacteria for Their Ability to Produce Biogenic Amines (Histamine and Tyrosine). *Int. J. Food Sci. Technol.* **2011**, *46*(10), 2062–2069. DOI: 10.1111/j.1365-2621.2011.02717.x.
[205] Savini, V.; Gherardi, G.; Marrollo, R.; Franco, A.; De Araujo, F. P.; Dottarelli, S.; Fazii, P.; Batiisti, A.; Carretto, E. Could β-hemolytic, Group B *Enterococcus Faecalis* Be Mistaken for

Streptococcus Agalactiae? *Diagn. Microbiol. Infect. Dis.* **2015**, *82*(1), 32–33. DOI: 10.1016/j.diagmicrobio.2014.12.005.

[206] Papaparaskevas, J.; Houhoula, D. P.; Papadimitriou, M.; Saroglou, G.; Legakis, N. J.; Zerva, L. Ruling Out *Bacillus Anthracis*. *Emerg. Infect. Dis.* **2004**, *10*(4), 732–735. DOI: 10.3201/eid1004.030544.

[207] Johnson, B. T.; Mayo, J. A.; Jeansonne, B. G. Beta-Hemolytic *Streptococci* and Other Beta-Hemolytic Organisms in Apical Periodontitis and Severe Marginal Periodontitis. *Endod. Dent. Trauma.* **1999**, *15*(3), 102–108. DOI: 10.1111/j.1600-9657.1999.tb00764.x.

[208] Linke, B.; Schreiber, Y.; Picard-Willems, B.; Slattery, P.; Nüsing, R. M.; Harder, S.; Geisslinger, G.; Scholich, K. Activated Platelets Induce an Anti-Inflammatory Response of Monocytes/Macrophages through Cross-Regulation of PGE2 and Cytokines. *Mediators Inflamm.* **2017**, *2017*, 1463216. DOI: 10.1155/2017/146321.

[209] Azizpour, K.; van Kessel, K.; Oudega, R.; Rutten, F. The Effect of Probiotic Lactic Acid Bacteria (LAB) Strains on the Platelet Activation: A Flow Cytometry-Based Study. *J. Probiotics Health*. **2017**, *5*(3), 1–5. DOI: 10.4172/2329-8901.1000185.

[210] Zhou, J. S.; Rutherfurd, K. J.; Gill, H. S. Inability of Probiotic Bacterial Strains *Lactobacillus Rhamnosus* HN001 and *Bifidobacterium Lactis* HN019 to Induce Human Platelet Aggregation *in Vitro*. *J. Food Prot.*. **2005**, *68*(11), 2459–2464. DOI: 10.4315/0362-028X-68.11.2459.

[211] Korpela, R.; Moilanen, E.; Saxelin, M.; Vapaatalo, H. *Lactobacillus Rhamnosus* GG (ATCC 53103) and Platelet Aggregation *in Vitro*. *Int. J. Food Microbiol.* **1997**, *37*, 83–86.

[212] Johansson, D.; Shannon, O.; Rasmussen, M. Platelet and Neutrophil Responses to Gram Positive Pathogens in Patients with Bacteremic Infection. *PLoS ONE*. **2011**, *6*(11), e26928–e26928. DOI: 10.1371/journal.pone.0026928.

[213] Shannon, O.; Mörgelin, M.; Rasmussen, M. Platelet Activation and Biofilm Formation by *Aerococcus Urinae*, an Endocarditis Causing Pathogen. *Infect. Immunol.* **2010**, *78*(10), 4268–4275. DOI: 10.1128/IAI.00469-10.

[214] Rasmussen, M.; Johansson, D.; Söbirk, S. K.; Mörgelin, M.; Shannon, O. Clinical Isolates of *Enterococcus Faecalis* Aggregate Human Platelets. *Microb. Infect.* **2010**, *12*(4), 295–301. DOI: 10.1016/j.micinf.2010.01.005.

[215] Pokhrel, P.; **2015**. Deoxyribonuclease (Dnase) Test-Principle, Uses, Procedure, Result, Interpretation, Quality Control, Examples and Limitations. http://www.microbiologynotes.com/deoxyribonuclease-dnase-test-principle-uses-procedure-result-interpretation-quality-control-examples-and-limitations/ (accessed Aug 14, 2017).

[216] Gupta, H.; Malik, R. K. Incidence of Virulence in Bacteriocin-Producing Enterococcal Isolates. *Le Lait*. **2007**, *87*, 587–601. DOI: 10.1051/lait:2007031.

[217] Acharya, T.; **2014**. Deoxyribonuclease (Dnase) Test: Principle, Procedure and Results. https://microbeonline.com/deoxyribonuclease-dnase-test-principle-procedure-results/ (accessed Dec 26, 2017).

[218] Sieladie, D. V.; Zambou, N. F.; Kaktcham, P. M.; Cresci, A.; Fonteh, F. Probiotic Properties of *Lactobacilli* Strains Isolated from Raw Cow Milk in the Western Highlands of Cameroon. *Innov. Rom. Food Biotechnol.* **2011**, *9*, 12–28.

[219] Acharya, T.; **2014**. Gelatin Hydrolysis Test: Principle, Procedure and Expected Results. https://microbeonline.com/gelatin-hydrolysis-test-principle-procedure-expected-results/ (accessed Dec 24, 2017.

[220] Guneser, M. B.; Eldeniz, A. U. *Enterococcus Faecalis* on Adhesion to Dentin after Irrigation with Various Endodontic Irrigants. *Acta Biomater. Odontol. Scand.* **2016**, *1*(2), 144–149. DOI: 10.1080/23337931.2016.1256212.

[221] Anderson, A. C.; Jonas, D.; Huber, I.; Karygianni, L.; Wölber, J.; Hellwig, E.; Arweiler, N.; Vach, K.; Wittmer, A.; Al-Ahmad, A. *Enterococcus Faecalis* from Food, Clinical Specimens, and Oral Sites: Prevalence of Virulence Factors in Association with Biofilm Formation. *Front. Microbiol.* **2006**, *6*(1534), 1–14. DOI: 10.3389/fmicb.2015.01534.

[222] Beecher, D. J.; Wong, A. C. Cooperative, Synergetic and Antagonistic Haemolytic Interaction between Haemolysin BL, Phosphotidycholine, Phospholipase C and

Sphingomyelinase from *Bacillus Cereus*. *Microbiol.* **2000**, *146*, 3033–3039. DOI: 10.1099/00221287-146-12-3033.
[223] Titball, R. W.;. Bacterial Phospholipids C. *Microbiol. Rev.* **1993**, *57*, 347–366.
[224] Bhat, A. R.; Irorere, V. U.; Bartlett, T.; Hill, D.; Kedia, G.; Morris, M. R.; Charalampopoulos, D.; Radecka, I. *Bacillus Subtilis* Natto: A Non-Toxic Source of poly-γ-glutamic Acid that Could Be Used as a Cryoprotectant for Probiotic Bacteria. *AMB Expr.* **2013**, *3*(36), 1–9. DOI: 10.1186/2191-0855-3-36.
[225] Georgescu, M.; Gheorghe, I.; Curutiu, C.; Lazar, V.; Bleotu, C.; Chifiriuc, M. C. Virulence and Resistance Features of *Pseudomonas Aeruginosa* Strains Isolated from Chronic Leg Ulcers. *BMC Infect. Dis.* **2016**, *16*(Suppl 1), 92. DOI: 10.1186/s12879-016-1396-3.
[226] El-Baz, R.; Rizk, D. E.; Barwa, R.; Hassan, R. Virulence Factors Profile of *Staphylococcus Aureus* Isolated from Different Clinical Sources. *N. Egypt. J. Microbiol.* **2016**, *43*, 126–144.
[227] Rijal, N.; **2015**. Nagler Reaction (Lecithinase Test): Principle, Procedure, Results and Limitations. https://microbeonline.com/nagler-reaction-lecithinsae-test-principle-procedure-results-limitations/ (accessed Dec 20, 2017).
[228] Sharaf, E. F.; El-Sayed, W. S.; Abosaif, R. M. Lecithinase-Producing Bacteria in Commercial and Home-Made Foods: Evaluation of Toxic Properties and Identification of Potent Producers. *J. Taibah Univ. Sci.* **2014**, *8*(3), 207–215. DOI: 10.1016/j.jtusci.2014.03.006.
[229] Teramu, E.; Shimura, S.; Karasawa, T. *Clostridium Tetani* Is a Phospholipase (Lecithinase) Producing Bacterium. *J. Clin. Microbiol.* **2005**, *43*(4), 2024–2025. DOI: 10.1128/JCM.43.4.2024-2025.2005.
[230] Ermolaeva, S.; Karpova, T.; Novella, S.; Wagner, M.; Scortti, M.; Tartakovskii, I.; Vazquez-Boland, J. A. A Simple Method for the Differentiation of *Listeria Monocytogenes* Based on Induction of Lecithinase Activity by Charcoal. *Int. J. Food Microbiol.* **2003**, *82*(1), 87–94.
[231] Macfarlane, G. T.; Gibson, G. R. Formation of Glycoprotein Degrading Enzymes by *Bacteroides Fragilis*. *FEMS Microbiol. Lett.* **1991**, *77*, 289–294. DOI: 10.1111/j.1574-6968.1991.tb04363.x.
[232] Abe, F.; Muto, M.; Yaeshima, T.; Iwatsuki, K.; Aihara, H.; Ohashi, Y.; Fujisawa, T. Safety Evaluation of Probiotic Bacteria Bifidobacteria by Analysis of Mucin Degradation Activity and Translocation Ability. *Anaerobe*. **2010**, *16*, 131–136. DOI: 10.1016/j.anaerobe.2009.07.006.
[233] Ruas-Maldiedo, P.; Gueimonde, M.; Fernández-Garcıá, M.; de Los Reyes-Gavilán, C. G.; Margolles, A. Mucin Degradation by *Bifidobacterium* Strains Isolated from the Human Microbiota. *Appl. Environ. Microbiol.* **2008**, *74*(6), 1936–1940. DOI: 10.1128/AEM.02509-07.