Using *Gro*EL (*hsp*60) for a Specific Quantification of Lactobacilli by Quantitative Real-Time PCR

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Für meine Eltern

Science, never rests. It is never satisfied; it is everlastingly thirsting for improvement, which the absolute ignores.

Victor Hugo, William Shakespeare, Part I Book 3, Art and Science

The work of the thesis had been performed from February 2010 until January 2014 at the Insitute of Microbiology and Epizootics at the Department of Veterinary Medicine at the Freie Universität Berlin. My thesis was supervised by Prof. Dr. Lothar H. Wieler and Prof. Dr. Rupert Mutzel.
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Table of Contents

List of Abbreviations

3-HPA	3-hydroxypropionaldehyde, Reuterin
454	Sanger or pyrosequencing
AG	Aktiengesellschaft
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATCC	American Type Culture Collection
BD	Becton Dickinson
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
bp	basepair(s)
CA, USA	California, United States of America
cfu / CFU	colony forming units
CHOC-agar	chocolate blood agar
Chr. Hansen	Christian Hansen
CNS	central nervous system
COL-agar	Columbia blood agar
CT, USA	Connecticut, United States of America
C_T	cycle treshold
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
ds	double-standed
DSM	Deutsche Sammlung von Mikroorganismen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority

e.g.	for example
FAO	Food and Agriculture Organization of the United Nations
FAM	6-carboxyfluorescein reporter
FDA	US Food & Drug Administration
FISH	Fluorescense *in-situ* hybridization
For / FOR / F	forward primer
FOSHU	Food for Specified Health Use
FTC	US Federal Trade Commission
FTIR	Fourier Transformation Infra-Red spectroscopy
GE	Genome Equivalents
GI tract	gastro intestinal tract
GRAS	Generally Recognized As Safe
h	hour(s)
HMI	Humana Milchindustrie GmbH, Everswinkel
IA, USA	Iowa, United States of America
IBMT	Fraunhofer-Institut für Biomedizinische Technik
IL-6,-10,-12	Interleukin(s)
IMT	Institut für Mikrobiologie und Tierseuchen
Inc.	Incorporation
IR	infra-red
ITS	intergenic spacer
LAB	Lactic Acid Bacteria
LBS	*Lactobacillus* Selection (Agar)
Ltd.	Limited
MA, USA	Massachusetts, United States of America
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry
Mb	mega basepairs

min	minute(s)
MLST	Multilocus Sequence Typing
MRS	de Man, Rogosa and Sharpe agar
n	number of tested individuals
NGS	next generation sequencing
No	Number
n.t.	not tested
PA, USA	Pennsylvania, United States of America
PABA	p-aminobenzoic acid
PCR	Polymerase Chain Reaction
PFGE	Pulse-Field-Gel-Electrophoresis
qPCR	quantitative real-time Polymerase Chain Reaction
R^2	correlation coefficient
RAPD	Randomly Amplified Polymorphic DNA
rDNA	ribosomal DNA
Rev / REV / R	reverse primer
RFLP	Restriction Fragment Length Polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
rT-qPCR	reverse Transcription quantitative real-time Polymerase Chain Reaction
s	second(s)
S1 / S2	Supplement 1 / Supplement 2
S-layer	surface layer
SDS	Sodium Dodecyl Sulfate
SFB	Collaborative Research Centres, Deutsche Forschungsgesellschaft (DFG)
SMRT	single-molecule real-time sequencing

SOLiD	Sequencing by Oligonucleotide Ligation and Detection
sp.	Species
spp.	Species
SSCP	Single-Strand Conformation Polymorphism
SSR	Single Sequence Repeats
subsp.	subspecies
TE	Tris-EDTA buffer
TGGE	Temperature Gradient Gel Electrophoresis
™	Trademark
TNF-α	Tumor necrosis factor alpha
Tris	Tri-sodium citrate di-hydrate
UHTS	Ultra-High-Throughput-Sequencing
US / U.S.	United States
USA	United States of America
WHO	World Health Organization
WGS	Whole Genome Sequencing
ZIM	Zentrales Innovationsprogramm Mittelstand
®	registered
~	approximately
<	less than

1. Introduction

1.1 Definition of Probiotics

1.1.1 History of utilizing Probiotics for Fermentation

Early in the history of mankind, lactose as a main sugar in milk was toxic to adults who could not produce lactase to decompose it. Thus, – except for children – milk could not be used as a nutrient-rich medium for the human diet. Throughout time, mutations conferring lactose tolerance accumulated in populations using milk as a food source and additionally, man has learned the art of reducing lactose to make milk a source of food. 11,000 years ago, people in the Middle East found ways to reduce the lactose level of milk products by fermenting milk into cheese or yoghurt. Similarly in Europe – nearly 7,000 years ago – the technique of separating milk from liquid whey was performed by using dotted clay pottery with small holes. Fragments of this pottery have been exposed by Peter Bogucki at a Stone Age-era site in central Poland. In 2011, Mélanie Roffet-Salque, a geochemist, analyzed the pieces and detected several remains of fatty acids of milk origin. Thus, these pottery remains represent the oldest cheese-making facilities to date worldwide (see [1] for review). Milk is an ideal medium for spoilage bacteria, molds, fungi and yeast to grow in [2, 3]. Even in production and post-manufacturing, environmental bacteria enter milk products [4]. Thus, storing raw milk is difficult due to the spoilage activity of several bacteria growing in a rich medium containing lactose and fat [5]. Before knowing about pasteurization, it was a challenge for humans to preserve milk: this was tested in several non-planned approaches, both by trial and error or pragmatic observation [6]. Millennia later, utilizing bacteria in a controlled way to preserve milk and other products such as vegetables was discovered [7]. Accidentally, useful bacteria were sticking to pots and tanks by secreted exopolysaccharides [6]. Thus, they were the first organisms encountered by milk when it entered the jars, starting the fermentation activity and preventing bacterial spoilage by their acid production [6, 8]. This exposing efficacy makes it difficult for spoilage bacteria to grow in an acidic environment [8, 9].

In the early stages of food fermentation, humans had to differentiate between pathogenic and food spoiling bacteria and beneficial cultures to perform food

fermentation utilizing severe mammalian milk sources such as cow milk, buffalo, goat, yak or sheep milk [6, 10].

Nowadays, a huge diversity of lactic acid bacteria (LAB) is known that are well adapted to milk as a preferred medium to grow in [11]. These bacteria are oxygen tolerant and use lactose as their source of energy [12].

In Egypt and other countries of the African continent, thousands of different LAB strains are used daily to ferment milk for domestic usage [10]. Even within a village or town, several non-analyzed mixtures of LAB are used for the preparation of fermented milk products [10]. Indeed, these mixtures have mostly been used for generations. For preparation, milk is warmed up, blended with a small amount of yoghurt, mixed up and stored at a warm place for incubation [6]. For instance, in Mongolia, a milk mixture is put in a bag of gastric stomach and placed in the sun [6].

Fermentation abilities of probiotic bacteria were also found to be useful and effective in the preservation of vegetables such as cucumber, olives, sauerkraut, Korean kimchi or red cabbage [13, 14]. Thus, this enables the storage of such products for periods in which they are not available, caused by seasonal reasons.

1.1.2 Definition of Probiotics

In 1907, Ilya Illitch Metchnikoff, a Ukrainian physiologist, was the first among those describing the beneficial effect of bacteria to improve human health [6, 15]. He observed that Parisians were dying earlier than Ukrainians and hypothesized that there are protective reasons why Ukrainians were living longer [6]. This finding was published in his book “The prolongation of life” [15]. Its basic concept was called “replacement of harmful microbes by useful microbes” [16]. Thus, at the end of his book, he speculated that microbes of the gut are manipulated by food and “good bacteria”, showing an excluding activity towards pathogenic bacteria [6].

The term probiotic was first used by Kollath (1953) in combination with several organic and inorganic compounds restoring the health of malnourished patients [17, 18]. The origin of the word “probiotic” is a combination of a Latin preposition pro ("for") and a Greek adjective of βίος (bios, "life")” [19]. After some redefinitions, Parker (1974) postulated that not only microbial organisms have an effect; moreover,

other substances such as antimicrobial pharmaceuticals or food composition might influence the intestinal microbial flora [20, 21].

In the absence of a precise definition of probiotic bacteria, Salminen et al. (1999) rephrased that probiotics have to not be viable when dispensed for having a health beneficial effect [22]. This was a new approach in defining probiotics. In addition, they defined probiotics as "viable microbial cultures balancing the microflora in the intestine in preventing or correcting microbial dysfunctions" [22].

FAO/WHO defined probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" [23]. In addition, they restricted its scope discussing "live microorganisms which, when consumed in adequate amounts as part of food[1], confer a health benefit on the host", by adding a footnote "[1]Water is included as a food." [23]. This restriction enunciated that this definition applies for probiotics being used in food only by an "exclude reference to term biotherapeutic agents, and beneficial microorganisms not used in food" [23]. Hence, initiating studies on healthy people are needed to determine the potential of probiotic bacteria in exerting health beneficial effects.

In addition, official FAO/WHO definition focuses on probiotics which are viable [23]. Viability is often closely connected to the cultivability of the used strains, which might temporary become lost although strains are still viable [24]. This phenomenon is caused by the physiological state that live bacteria are in (e.g. refrigerated milk products and beverages), despite not being cultivable in this state [24].

The US Food & Drug Administration (FDA) (2006) defined probiotics used in feed as "direct fed-microbials" and "live biotherapeutics" when utilized in human drugs [25, 26]. Based upon this definition, probiotics have to be viable when administered to the host for having a beneficial effect.

In Asia contrary to the FAO/WHO definition, probiotics are defined and cover viable and non-viable cells of probiotic bacteria demonstrating health beneficial effects on the host [22]. However, Lahtinen et al. (2006) showed that even dead cells can have an effect [27]. Several promising studies utilizing non-viable heat-killed or otherwise inactivated cells have demonstrated their effectiveness by cell wall components and even by fragments of the nucleotide sequence [22, 28].

1.1.3 Genera and Species

Vast amounts of diverse species of different genera with probiotic effects are known. The most frequently used species in dairy products belong to the genera *Lactobacillus* (*L.*) or *Bifidobacterium* (*B.*) [29]. Other common probiotic species are *Streptococcus* (*St.*) *thermophilus*, *Escherichia* (*E.*) *coli*, *Lactococcus lactis*, *Enterococcus faecium* or spore-forming *Bacillus toyonensis* [30, 31]. Less common species include *Propionibacterium*, *Leuconostoc* (*Leuc.*), *Pediococcus* (*Ped.*) or yeasts such as *Saccharomyces* (*Sa.*) *cerevisiae* or *Sa. boullardii* [32, 33].
The best human efficacy data in preventing or treating diseases have been established for *L. rhamnosus* GG (Valio®), *Sa. cerevisiae* Boullardii (Biocodex), *L. casei* Shirota (Yakult®) and *B. animalis* Bb-12 (Chr. Hansen®) [34]. These strains are ideal for dealing with lactose malabsorption, rotaviral and antibiotic-associated diarrhea and diarrhea caused by *Clostridium* (*C.*) *difficile* [34].

1.1.4 Probiotic Products

Most probiotic species are known to induce the fermentation process in different foods. Thus, many or even most fermented foods contain co-cultures or starter cultures such as lactobacilli, lactococci, streptococci, enterococci or yeasts [35, 36]. They have been used for centuries, involving known safety standards and health beneficial effects [37].
The optimal environmental food conditions are prepared by starter cultures. The utilized strains are fermenting carbohydrates such as lactose, glucose and fructose to lactic acid, enabling facultative probiotic anaerobes to widen or adopt fermentation [38].
Many species of several genera are well known as starting cultures in the fermentation process of milk products [39]. Species such as *L. acidophilus*, *St. thermophilus* or *L. delbrueckii* subsp. *bulgaricus* are used for yoghurt fermentation [39]. It has been shown that different combinations of these three species result in differential fermentation time and considerable differences in their taste [39]. For instance, normal yoghurt fermentation is carried out at 40-45 °C for 2.5 h utilizing *L. delbrueckii* subsp. *bulgaricus* and *St. thermophiles* [39]. Instead of this, mild yoghurt

fermentation needs up to 6 to 8 h, using *St. thermophiles* and *L. acidophilus* [39]. While some probiotic strains can be inoculated in combination with traditional starters, in stirred yoghurts they are added after fermentation to maintain their viability [40].

L. delbrueckii subsp. *bulgaricus* is very fastidious towards the medium as it shows abnormal morphologies if nutrients or minerals are missing [41].

By contrast, *L. acidophilus* is a robust species possessing properties that are important for its survival in the crude environmental gut conditions [42]. It is able to excrete mucus-binding proteins, it has an enormous acid tolerance level and is least affected by bile salts due to surface reduction [41]. Although it is slow growing in acidophilus milk, it promotes beneficial and therapeutic properties [43].

Additionally, probiotic bacteria are also used for matured and fresh cheese preparation [40]. The flavor and taste of cheese is influenced by a mixture of strains being used for the fermentation and ripening process [44].

As a result of recent investigations aimed towards improving the viability of the used strains, species have been successfully added to non-fermented products such as ice-cream, dry infant formulations and powders, cereal products, juices, probiotic straws and chocolate [37, 40].

Nevertheless, some genera of LAB are used for vegetable fermentation, such as lactobacilli, leuconostocs, and pediococci [45]. The most frequently used LAB species includes *Leuc. mesenteroides*, *Ped. cerevisiae*, *L. brevis* and *L. plantarum* [45]. For instance, sauerkraut is made by an initial fermentation started by the heterofermenative activity of *Leuc. mesenteroides*, which amongst others produces carbon dioxide [45]. After eight days, this environment promotes the growth of facultative anaerobes such as *L. plantarum*, which is associated with raw cabbage [45]. Due to anaerobic fermentative conditions, aerobes such as fungi are not able to grow in this acidic environment (pH range 1.5 to 3.9) [45].

In animal nutrition probiotic species such as *L. plantarum* and *L. buchneri* are used for silage preparation [46]. In this case, *L. buchneri* is used for forage preservation [46]. The most dominant probiotics being used in animal feed are *Bacillus* spp. spores [47]. They are more robust than lactobacilli due to their ability to administer their spores as feed additives [48].

1.2 Lactic Acid Bacteria

1.2.1 Definition

The term lactic acid bacteria (LAB) generally refers to “milk-souring organisms” [49]. LAB are a group of several Gram positive bacteria combined within morphological, metabolic and physiological characteristics [49]. Members are generally non-spore forming, non-respiring cocci or rods, producing lactic acid as a major product in the fermentation of carbohydrates [49]. Moreover, they even have the ability to grow under high salt conditions and having a huge acid and alkaline tolerance level (pH range between 3 and 8) [50]. Milk-souring and other bacteria are able to produce lactic acid, as described by Orla-Jensen (1919) [49, 51]. Nowadays, the criteria published by Orla-Jensen, such as morphology of the cells, characteristics of the glucose fermentation pathway, growth temperatures and sugar utilization, are used for the classification of LAB [49]. However, these identification methods are time-consuming, labor-intensive and less usable for daily routine diagnostics. In recent years, molecular tools such as rRNA sequence analysis or whole genome sequencing (WGS) have become widely utilized to characterize LAB and other bacteria [30, 49].

1.2.2 Potentials of LAB

The lactobacilli possess small genomes in comparison to other genera of LAB [52]. Genome size varies between 1.8 and 3.3 Mb and a G+C content of 33 to 51 % [53]. Species with small genomes show a high adaptation to carbohydrate-rich environments, as opposed to more eclectic species with large genomes [54]. For instance, members of the genus *Lactobacillus* have lost their ability to form spores, as their ancestor *Bacillus* is able to do [54]. During the course of evolution, the members of the genus *Lactobacillus* might have lost up to 1,000 genes and exhibit a higher number of pseudogenes [54, 55]. Lost genes are related to carbon metabolism or amino acid transport and metabolic systems least needed in milk products [55]. For instance, *L. helveticus* is dedicated to milk products [56]. Furthermore, some species are adapted to a wide range of niches, such as *L. rhamnosus*, which is used in several products [57]. It has been isolated from diverse ecological habitats such as

the human intestinal tract, vaginal and oral cavity and cheese [57]. This species shows a wide range of metabolic capacities, including its adaptation to survive in the gastro intestinal (GI) tract [57]. Genes concerning bile resistance, anti-microbial activity and mucus-binding pili appear to be active for adoptive survival in the harsh conditions of the GI tract [57].

Gene analysis of several lactobacilli have shown that adaptation to diverse ecological niches was carried out by an acquisition of genes via horizontal gene transfer [57]. For instance, *L. rhamnosus* might have acquired the *spa*CBA pili gene cluster by gene transfer, enabling it to bind to the human mucus layer to exert immune modulating effects [57, 58]. Due to this adaptation process, several gene clusters appear to be lost during the course.

Despite this loss of genes within genera represented in LAB, members are adapting rapidly to new ecological environments represented in different food and feed sources [59]. Additionally, some genera (*Streptococcus*, *Lactococcus*, *Enterococcus*, *Carnobacterium*) are harboring species that are well-known as being pathogenic, causing diverse diseases [49].

1.2.3 Genus *Lactobacillus*

Genus *Lactobacillus* is the largest group within LAB, being part of the phylum *Firmicutes* [60]. Different species represent a large heterogeneity and variety of phenotypic, biochemical and physiological properties [61]. This genus is adaptive to diverse habitats such as carbohydrate-rich environments of dairy products, persisting in the microbial-rich intestine and finding its natural niches in plant and soil environments [37, 62]. Lactobacilli are Gram positive, variable in morphology, non-sporulating and aerotolerant or anaerobic [37, 41]. In total, their genome presents a low G+C content, although its upper level reaches 59.2 % [50, 63]. The optimal growth conditions of lactobacilli are between 30-40 °C and a pH value of 5.5 to 6.2, respectively [50].

In humans, they persist in the GI tract, the urogenital tract, and are located in the oral cavity, causing dental plaque, and represent skin microbial flora [37]. In healthy women, lactobacilli are part of the vaginal microbiomes responsible for maintaining the acidic environment [64]. Lactobacilli in mothers' breast milk have a high

probiotic impact in terms of developing and initializing the neonatal microbiota [65]. Species such as *L. fermentum*, *L. salivarius*, *L. rhamnosus* and *L. gasseri* have been isolated from human milk [65-67]. These species appear to be delivered by milk and produce bacteriocines exerting antimicrobial effect towards pathogenic bacteria [66, 68]. They are involved in reducing the pH value through their fermentation activity [66, 68].

Despite possessing a preservation effect on food, different species of the genus *Lactobacillus* are known as food contaminants [69]. For instance, cereal grains have been used to prepare home-made beer containing LAB being integrated in lactic fermentation [69]. In industrial breweries, *L. brevis* is the most unrequested contaminant, causing beer spoilage [70]. In addition, *L. kunkeei* ATCC 700308 was isolated in 1997 as having wine contaminant abilities [71]. Even the starter culture activity of *L. sake* causes a more rapid bane of fermented and sliced meat products before reaching their expiring date [72].

A large number of dietary probiotic species of the genus *Lactobacillus* are assigned as being GRAS (Generally Recognized As Safe), caused by their long history of being added to food without any risk to health [73]. Indeed, this is why lactobacilli – which are more robust during manufacturing and storage than bifidobacteria – are the most interesting and used genus in food microbiology and human nutrition [74, 75].

1.2.4 Multiplicity of the Strains

A large diversity of probiotic strains is used in probiotic products, which have positive effects on the host. However, it has been found that these health-promoting effects depend on the strains and their unique biochemical characteristics [76, 77]. Despite being widely described in literature, these beneficial effects are not species- or genus-specific [34]. Thus, no single probiotic strain is able to offer all health beneficial effects as shown by different strains of the same species [34].

By knowing that promoting effects are strain-specific, strain selection is fundamental and most essential to maximize their potential for the manufacturing process and their health benefits [76].

Herein, it is necessary to analyze a strain's molecular abilities to characterize its advantages and disadvantages in fermentation and regarding its beneficial side. To

conduct screening of the strains, rapid techniques have to be used. Within recent years, costs for WGS or high throughput sequencing have steadily decreased, and these technologies are now being commonly used for structural and genetic analysis [78].

Several investigations have revealed an increasing effect of probiotic bacteria administered in a mixture instead of single usage [79]. Nonetheless, the triggering effects remain unclear in terms of increasing the impact when strains are used in a composition of different probiotic bacteria. This might be caused by synergetic effects due to interaction activity between species or by higher probiotic cell dosages established in the product [79].

For instance, *L. acidophilus* NCFM showed good results in reducing the incidence of colon cancer in rats consuming a meat-based diet [34, 80]. In addition, the same strain reduces the activity of fecal enzymes such as ß-glucuronidase, nitroreductase and azoreductase in humans and prevents urogenital tract infections [34, 81, 82]. Other strains of the same species increase the production of IL-6, IL-10, IL-12 and TNF-α [83].

1.3 Methods of Species Identification

The identification and classification of bacteria is the initial point to characterize microbial activities and the properties of microorganisms [24]. An accurate and reliable strain identification is necessary to assure safety and efficacy of the used strains, which is essential for utilizing it as food and feed additives [84, 85]. Knowledge about each strain is important to formulate a mixture of starter cultures and other probiotic strains to assure efficacy and safety [86].

In recent decades, several molecular identification techniques have been designed to replace error prone and time-consuming phenotypic methods, such as screening the morphology or physiological needs of strains. DNA-DNA hybridization has evolved as the most important method to characterize bacterial identification. It has been claimed that two strains belong to one species if DNA-DNA relatedness is greater than or equal to 70 % [24].

In phylogenetic identification approaches, sequence analysis of 16S rRNA is widely used for the identification of bacteria in food industry, in comparison to other target genes [87]. Herein, microorganisms that share a 16S rRNA gene homology of more than 97 % are supposed to be members of the same species [24].

Thus, in 2002 FAO/WHO published a second document focusing on techniques for the correct identification of probiotic strains [88]. Herein, a scheme for a correct characterization of bacteria has been summarized to define microorganism as 'probiotic' (Table 1).

Table 1 Characterization steps for qualifying bacterium as probiotic according to FAO/WHO guidelines [84, 88].

Identification	Strain identification by phenotypic and genotypic methods at genus and species level
	Deposit strain in international strain collection
Safety assessment	In vitro tests and/or animal models
	Phase 1 human study
	Antibiotic resistance pattern
Efficacy assessment	In vitro tests
	Animal studies
	Double-blind, randomized, placebo-controled (DBPC) phase 2 human trial or other appropriate design with sample size and primary outcome appropriate for determining whether the strain/product is efficacious
	Preferable a second independent DBPC study to confirm results

However, despite phenotypic tests being time- and labor-intensive, FAO/WHO experts advised that they have to be performed before proceeding towards testing bacteria by molecular biological methods such as DNA-DNA hybridization, 16S rRNA sequencing or RAPD-PCR profiling [84, 89].

Characterization starts with the correct manifestation of the bacterium at strain level due to beneficial properties, which are strain-specific [90]. In addition, strain-specific characterization enables to collect epidemiological, safety and efficacy data, which

are needed in license, copyright, trademark and authority permission processes. However, FAO/WHO guidelines (2002) have limited effects on industry patterns [84, 88].

In order to meet the needs of species-specific identification, some other techniques such as DNA microarray analysis, temperature or denaturing gradient gel electrophoresis (TGGE or DGGE) are used [91]. Although DGGE offers a limited quantitative approach of bacterial cells, these methods should not be used as stand-alone techniques for the quantitative analysis of probiotic bacteria [92, 93]. Therefore, in addition, fluorescence *in situ* hybridization (FISH) or quantitative real-time PCR are used for quantifying LAB [94].

1.4 Probiotics and Health Claims

Probiotics are microorganisms that are thought to promote health beneficial effects and enhance well-being when administered in food [95]. Previously, health claim regulations had been established in Japan in association with the law based on Foods for Specified Health Use (FOSHU) of products having physiological functions on human health [24, 96]. Recently in 2006, European-wide regulations regarding health claims in foods assessing their standards in Regulation 1924/2006 were adopted by the European Parliament and the Council [24]. The main aims of the regulations on food are a high level of consumer protection, effective functioning of the internal market within the European Union, fair competition within food industry and stimulating and protecting innovations [24]. Thus, regulation (EC) No. 1924/2006 includes disclosing health-related aspects on functional food products in commercial communications regarding labeling, presenting, advertising, brand names and trademarks [24]. As a result, all health claims made on each product should provide no false or misleading information, should not challenge the nutritional aspects of the food they are in, or interfere with or excess total food consumption [24]. Nutritional facts of food products should be in line with nutritional recommendations that are part of a balanced diet [24].

However, health claims have been rejected or negatively advised by EFSA (European Food Safety Authority) due to inadequate taxonomic classifications [84, 97]. EFSA stated that beneficial effects such as changing the composition of white

cells in blood are not coequally a health promoting physiological effect [97]. Consequently, this was the only way that EFSA could react concerning health claims deliberated by companies interested in placing their products using health claim advertisements [97]. Within the European Union and covered by national law, it is difficult to indicate beneficial effects of probiotic bacteria within the prohibition to state medical claims that food products are preventing, treating and curing disease [97, 98]. However, this is lacking within the directive 2000/13/EC, because it has been shown that lemons cure scurvy and folic acid prevents neural tube defects [98, 99].

In 2010, the US Federal Trade Commission (FTC) focused on health claims used by Dannon® and Nestlé® in advertising probiotic products. Nevertheless, both companies faced difficulties in advertising their products in combination with drug-like claims without having consolidated Investigational New Drug (IND) supporting data [100]. Dannon® placed the information that Activia® prevents colds in children due to "strengthening the body's defenses" by speeding the transit time of food through the digestive tract [100]. On December 15th, 2010, this false claim was banned by the US Federal Trade Commission (FTC) due to the fact that the company did not provide human trial data in supporting the health claim [100].

Nestlé Healthcare Nutricion® was challenged by FTC regarding their advertisement of a probiotic beverage for children (BOOST Kid Essentials®) that prevents colds and flu by immunity boosting and helps to cure children from diarrhea [100]. Presumably, data of human studies were missing, which resulted in banning Nestlé®'s advertisement. On July 14th, 2010, Nestlé® announced that advertisement of this beverage would be rephrased as "providing complete nutrition for kids ages 1 to 13." [100].

These examples illustrate that companies are not resiling such regulatory authorities such as FTC. Their profit margins are much higher than for yoghurt or other milk products [97, 101]. For instance, the current purchasing price for Actimel® (3,49 €/l), Activia® (3,98 €/l) and Yakult® (7,66 €/l) in Germany is much higher than for normal yoghurt (0,98 €/l), although even natural yoghurts containing probiotic starter cultures possess more or less the same beneficial effects for the host [102]. It is an advantage for companies selling probiotic products that consumers are more focused and concerned on healthy food. The profit margins of probiotic products are high due to patents that are easy to obtain for unique bacteria instead of new vitamin or

mineral compositions added to food [97]. Thus, it is easy to produce a "new" bacterial strain by stressing and getting the most robust ones, which are still similar to common strains used in yoghurt fermentation to obtain a 'food grade status'. However, a newly "generated" strain is different enough to be patented [97]. Within trademark process, these "new" strains will get an imaginative name such as *L. casei Defensis* or Immunitas(s) (strain origin: *L. casei* DN-114001, Actimel®, Nestlé®) or Bifidus Acti Regularis® (strain origin: *B. lactis* DN-173 010, Activia®, Danone®) for marketing reasons [103].

1.5 Health Benefits

Several studies have shown that probiotics have single or multiple strain-specific mechanisms to influence humans or animals intestinal flora, increasing barrier functions, influencing the generating process of mucus layer or excluding pathogenic bacteria (Figure 1) [68, 90, 104, 105].

While some bacteria are of maternal origin, most commensal intestinal organisms colonize the gut in the first days of life [106]. Joshua Lederberg, who received the Nobel Prize in physiology in 1958, designated the intestine as a supra-organism harboring the microbiom as a dominant partner and our body as a minor one [6].

Probiotics persist in the lumen of the intestine and are able to influence the present bacteria by producing antimicrobial compounds such as bacteriocines or toxic conditions, they are reducing the pH level or by baneful compounds (Figure 1) [105, 107]. A major effect is closely connected to the microbiota of the host's gut. LAB are able to interfere with the microflora system by changing the composition or replacing missing or absent species [6, 108, 109]. Additionally, potential competitors are depressed in their local growth activity [6]. This effect has been observed by isolating the first probiotic strain *E. coli* Nissle 1917 from a soldier who escaped a *Salmonella* outbreak, making him resistant towards diarrhea caused by *Salmonella* [6, 110, 111].

Strompfová et al. (2013) demonstrated that a canine-derived *L. fermentum* strain has several health beneficial effects in healthy dogs [112]. In combination with inulin, a prebiotic supplement, a decrease of clostridia and an increase of *L. fermentum* occurred.

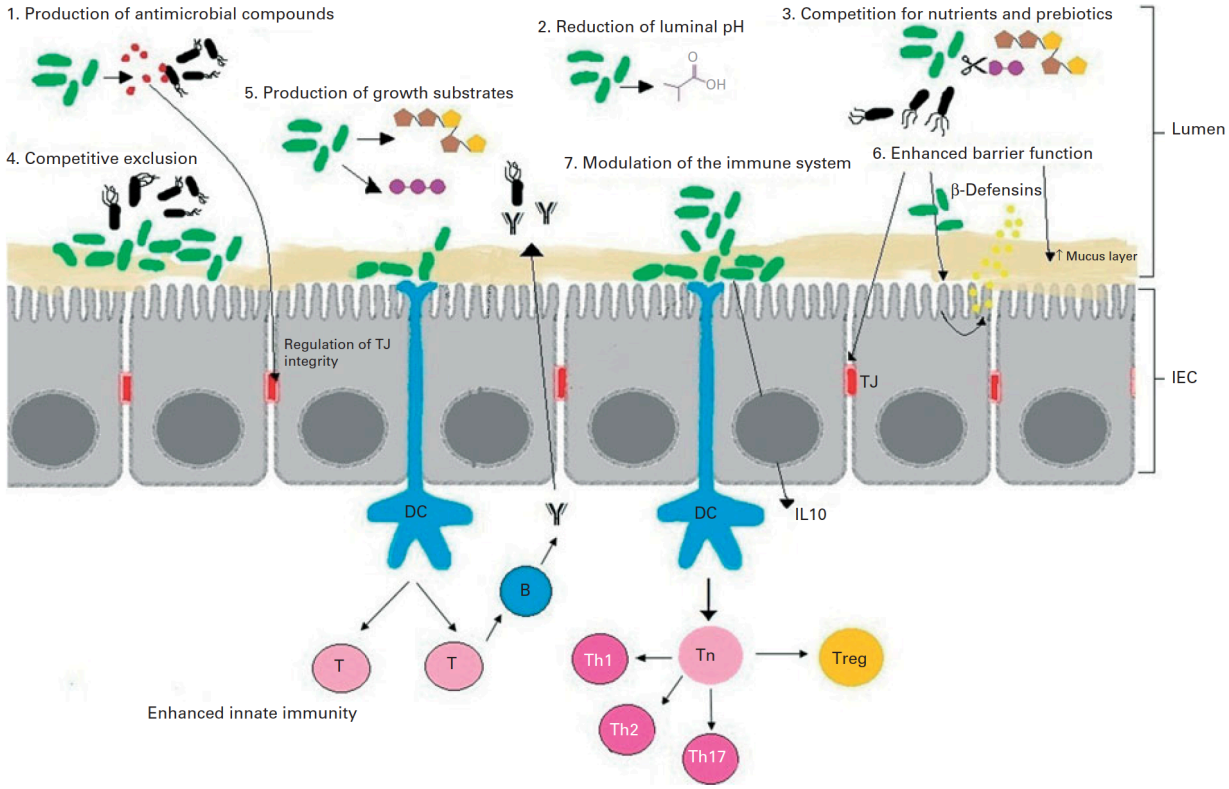

Figure 1 Beneficial effects and mechanisms of probiotic bacteria influencing gut microbiota and inducing conductive host responses: 1. Producing bacteriocines as antimicrobial compounds; 2. Reducing luminal pH by fermentation activity; 3. Competing for nutrients and prebiotics with pathogens; 4. Competing effect by excluding pathogens to adhere to epithelial cells; 5. Producing growth substrates such as vitamins, polysaccharides, metabolites and exopolysaccharides; 6. Enhancing intestinal barrier functions by effecting mucus and ß-defensin secretion, modulating cytoskeletal and tight junction protein phosphorylation; 7. Modulating the immune response; IEC, intestinal epithelial cells; DC, dentritic cells; TJ, tight junctions; B, B cells; T, T cells; Tn, naïve T cells; Treg, regulatory T cells. (from Power et al. 2013) [68].

This was positively supported by an increasing acidity caused by butyric, succinic, valeric and formic acid, respectively [113]. In humans, *L. fermentum* enables the production of fermenticin, a class IIa antimicrobial peptide, which was isolated from the vagina [114]. Herein, it inhibits various pathogenic bacteria, such as members of *Bacteroides*, *Mobiluncus*, *Gardnerella vaginalis*, staphylococci and streptococci causing vaginosis [114].

Lactobacilli and bifidobacteria are more or less stress tolerant, compete for epithelial binding sites and stimulate the immune system (Figure 1) [105, 115, 116]. It has been shown that some genes of gut cells are influenced by the activity of probiotic bacteria [117]. Additional amounts of probiotic bacteria compete for nutrients, prebiotics and other compartments being available, which are also preferred by pathogenic bacteria (Figure 1) [110]. Therefore, probiotic bacteria possess the ability to digest these prebiotics of food to ensure full energy exploitation by the host [118].

Nevertheless, probiotics also possess an effect on gut-brain axis activity and thereby influence the central nervous system (CNS) [106]. It has been demonstrated by Foster and McVey Neufeld (2013) that minor changes in microflora influence stress-related behavior [106]. Hence, commensals, probiotics and pathogenic bacteria of the gastrointestinal tract are likely to play a role in the activation of neural pathways and neurological signaling cascades [119]. Thus, the beneficial effects of probiotics and microbiota involve mental and health elements and might play an important role in daily life. Cryan and Dinan (2014) established the word “psychobiotics”, which represents probiotics’ effect on the gut-brain axis [120].

These beneficial effects not only refer to the GI or urogenital tract. Indeed, LAB are beneficial due to their ability to change the structure of milk protein by the fermentation process [6]. The reshaping process of the quaternary protein structure and hydrolyzing amino acids and peptides of milk proteins will finally avoid casein clotting in the human stomach [6]. Accordingly, this increases the digestibility of milk products.

1.6 Diseases Caused by Probiotics

Probiotics that are currently used have been long considered as safe [24]. Even manufactured mixtures of several probiotic strains are assured of being in line with the ideal probiotic properties (Table 2). However, new formulations and mixtures of different probiotic strains in diverse food or pharmaceutical administrations have to be tested and ensured as being safe, respectively [24]. Although many strains have the GRAS status, some cases are known in which probiotic bacteria cause infections in patients. For instance, patients with hepatitis or immunocompromised status have shown symptoms of probiotic infections [121].

Table 2 Properties of ideal probiotic strains (adapted from [68, 105, 122, 123]).

1. Non-pathogenic/non-inflammatory
2. Antimutagenic and anti carcinogenic properties
3. Resistant possessing, technological and storage conditions
4. Resistant to gastric and bile juices
5. Adherence to epithelium or mucus layer
6. Production of bacteriocines and metabolic activity
7. Persisting and surviving in the intestinal tract
8. Modulate pH value, immune response, influencing microbiota composition, producing inhibitory compounds

Besselink et al. (2008) found that probiotic prophylaxis severed to patients with acute pancreatitis does not reduce the risk of infectious complications [121]. Rather, probiotics increased the risk of mortality and should not be used in treating patients with acute pancreatitis [121].

In another case, a 24-year-old patient showed increased symptoms of sepsis caused by *L. rhamnosus*, which was previously administered preoperatively to an aortic valve replacement [124].

These two examples demonstrate that probiotics are likely to cause infections in patients with organ failure, with an immunocompromised status or dysfunctional gut barrier mechanisms [124]. Thus, the safety of probiotics has to be assured and guidelines for the labeling of functional foods regarding their potential risk should be thoroughly reviewed.

1.7 Abilities of Probiotics in Manufacturing Processes Regarding Stress and Safety

1.7.1 Selection Criteria of Probiotic Strains

Different criteria are used for the definition of an ideal probiotic strain. It has been shown that these strains are not of human origin, as mentioned by Antoine (2012) [6]. Instead of this, all microbes such as mammalian microbiota enter the host from environmental sources [125]. Other sources are microbes of the maternal gut, the vagina, from parental skin origin, breast milk, foods and fluids or neonates at maternity ward [125]. First, microbes of the mother's gut are predominantly the first bacteria to colonize the infant's intestine, explaining the availability of some well-adapted genera (bifidobacteria, lactobacilli, enterococci, *Enterobacteriaceae*) or species (*C. difficile*, *St. aureus*, *E. coli*) [125]. It should be noted that there are no known probiotics or microbes of human origin [6]. One clear reason is that microbes have been living on earth much earlier than mammals, who are born sterile [6].
In addition, several other criteria are important to define a strain as being a probiotic one. Most strains that are established in several dairy and non-dairy products have been described and tested within the following safety conditions (Table 2).
Furthermore, the selection of robust strains is important to confirm their viability standards in manufacturing process (Figure 2) [92].

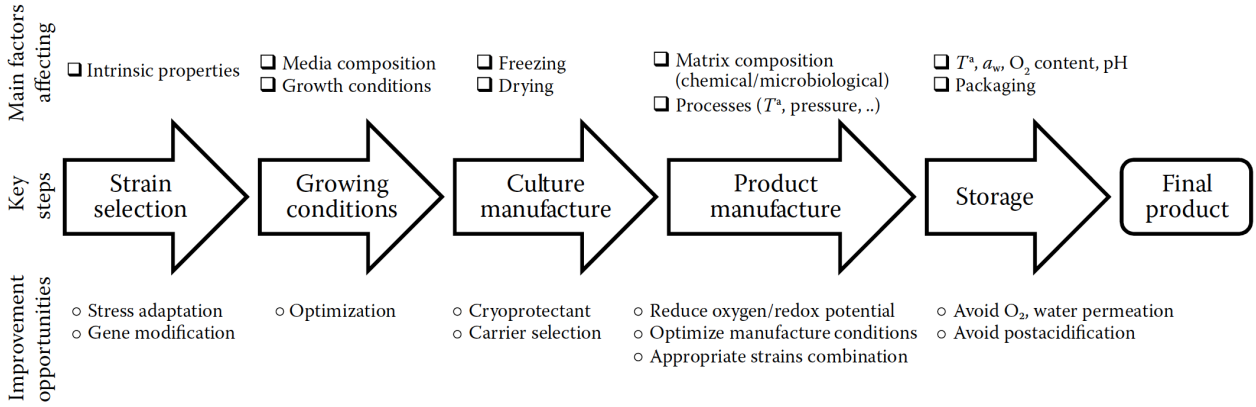

Figure 2 Process of product development with stable and robust LAB or strains of the genus *Bifidobacterium* [92].

1.7.2 Stress in Manufacturing and Storage

Manufacturing functional foods containing viable probiotic bacteria until the end of their expiring date is difficult and not without difficulties [92]. Some bacteria have been established as starter cultures, which easily tolerate increasingly acidic conditions that are established during fermentation [38]. The starters appear to possess good stability, viability and accessible to these conditions. Herein, starter cultures with a lack of postacidification are in favor of being used for the fermentation process [126]. Other bacteria of LAB or *Bifidobacterium* have been added to the same product, being less adapted and sensitive to environmental conditions such as decreased pH value, towards other microorganisms within the product's mixture, to temperature, oxygen or chemical conditions and high or low water activity [92]. All these parameters might influence the viability, stability and thereby efficacy and their beneficial effect of the added probiotic strains. Hence, precise knowledge of the manufacturing process, the selection of strains and strain mixtures are necessary to ensure increased viability and the retention of beneficial functionality during production and storage time (Figure 2) [127]. Grzeskowiak et al. (2011) demonstrated the influence of manufacturing and processing parameters on the strain properties [128].

Another possibility arises by adapting the used strains to stress by exposing them to sublethal environmental conditions such as heat, oxygen, acid, bile salts, freezing or dry conditions [92, 129]. It was shown that this strategy enhances the strain survival rate [130, 131]. Additionally, an increased tolerance level towards bile salts had an effect in more robustness of the strain towards low pH conditions (Figure 2) [132].

Besides the adaptation of strains to harsh conditions, different mechanical protection possibilities such as encapsulation have been established [133]. Materials such as starch, sucrose, trehalose or inulin are predominantly used (Figure 2) [134].

Genetic modification is feasible to increase the stress tolerance of strains [92]. Thus, strains of the genus *Lactobacillus* have been modified accordingly to enhance their ability to accumulate salts as compatible solutes [135]. However, freeze- or spray-drying are much more effective in terms of increasing the survival of probiotic bacteria in food formulations [136]. To enable bacteria being protected by freeze

drying cells in the stationary growth phase are centrifuged, resuspended in a cryprotectant medium, frozen and added into the product of choice [92].

Even the packaging process and the nature of packaging materials of foods containing probiotics significantly influence the survivality of the strains [137]. Manufacturers should avoid entry of oxygen into a product's matrix in the packaging area by providing a stable and modified controlled atmosphere [138]. However, even oxygen diffusing through the plastic yoghurt cup affects starter and non-starter bacteria of the product [139]. Therefore, the packaging of probiotic yoghurt in glass bins has been shown to prevent oxygen diffusion [35]. Additionally, adding carbon dioxide into milk reduces the risk of contamination in yoghurt manufacturing (Figure 2) [140, 141].

After being affected by product and storage conditions, strains face the challenge of reaching the GI tract. Hence, they have to be stable in harsh GI conditions caused by enzymes, an acidic pH value and by bile salts, which are a natural barrier against viable pathogenic bacteria [61]. Thus, this is a criteria for probiotic bacteria even being able to exert their beneficial effects within these environmental conditions [61].

Although different species and mixtures of different strains have been successfully added to different products, a reliable identification and quantification approach is still missing. At present, the quality management and quality assurance of dairy and non-dairy products have to be managed utilizing time-consuming and labor-intensive techniques, which have to be confirmed using an additional method in parallel. These approaches of daily diagnostics are costly and need to be exchanged by a validatable method, which enables both the identification and quantification of probiotic bacteria used in any product.

1.8 Aim of the Study

LAB are widely used in dairy and non-dairy products and are advertised as being health beneficial functional food that enhances well-being and promotes healthy life. Therefore, a correct identification of the used bacterial strains is important. Nevertheless, the amounts of probiotic bacterial cells play a major role in significantly offering health beneficial effects. While methods such as PCR or

MALDI-TOF MS often enable species or even strain-specific identification, they lack the ability to quantify detected species. For this purpose, a species-specific quantitative real-time PCR has been established to identify and quantify nine different species of the genus *Lactobacillus* (*L. acidophilus*, *L. brevis*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. helveticus*, *L. jensenii*, *L. johnsonii*, *L. reuteri*, *L. salivarius*) based on the *Gro*EL and *rec*A gene. It has previously been shown that these target genes work well within PCR approaches [142, 143].

Within the genus *Bifidobacterium*, two species were detectable and quantified by real-time PCR technique using the *clp*C target gene: *B. animalis* subsp. *lactis* and *B. bifidum*. The *clp*C target gene species-specificity has been demonstrated by Avershina et al. (2013) [144].

Within the project, several other identification tools have been used to assure strain identity of the acquired probiotic strains from different partners and the bacteria isolated from different products. Identification and strain assurance was achieved by molecular based techniques such as species-specific colony PCR and sequence analysis or by physiological testing utilizing API 50 CHL stripes from BioMérieux. However, the isolation of the bacteria from food origin is needed for some techniques, which requires additional time in identifying the strains at the species level.

The initial objective of this thesis was to establish an accurate detection and quantification approach of different probiotic species by real-time PCR in order to identify them in a culture-independent way within seven hours.

Finally, this technique offers a much faster routine diagnostic approach for charge testing within manufacturing process, as well as for authorities to assure quality standards of e.g. milk and meat products, pharmaceuticals or non-fermented products containing probiotics.

2. Publications

2.1 Species-specific Quantification of Probiotic Lactobacilli

2.1.1 Contribution to this Paper

I designed, structured and prepared the manuscript and the experiments described in the publication. In addition, I discussed and interpreted the results. The final manuscript version had been prepared by myself after discussing it with all the authors mentioned on the manuscript. BL helped within the process of preparing the experiments regarding plating and counting within the colony forming unit testings. MvN-R was responsible for the concept of the project and he drafted and revised the manuscript critically. MK was responsible for the concept of the project and he drafted and revised the manuscript critically. JM designed, structured and prepared the experiments regarding the identification of probiotic species by MALDI-TOF MS. In addition, he drafted and revised the manuscript critically for important intellectual content within the MALDI-TOF MS paragraph and has given final approval of the version to be published. LHW drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript, in structural arrangement of the paragraphs and he was responsible for the concept of the project. SG drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript, in adjusting the text and he was responsible for the concept of the project.
All authors read and approved the final manuscript.

2.1.2 Publication

Herbel, SR, Lauzat, B, von Nickisch-Rosenegk, M, Kuhn, M, Murugaiyan, J, Wieler, LH, Guenther, S (2013). Species-specific quantification of probiotic lactobacilli in yoghurt by quantitative real-time PCR. J Appl Microbiol 115(6): 1402-1410.

http://dx.doi.org/10.1111/jam.12341

2.1.3 Summary

Aims

Lactobacilli strains with probiotic effects have been widely used in dairy products such as yoghurts as well as in food additives and pharmaceuticals. Despite their successful commercial application the current species identification and quantification methods of the genus *Lactobacillus* are time-consuming and labor-intensive.

Methods & Results

In order to fulfill requirements of a robust quality management we have developed a quantitative real-time PCR assay based on the heat shock protein 60 gene (*hsp*60) for accurate identification and quantification of five commercially important *Lactobacillus* species. The developed assay allows an unambiguous species-specific detection of the species *L. acidophilus*, *L. brevis, L. delbrueckii* subsp. *bulgaricus, L. helveticus* and *L. reuteri* from bacterial cultures as well as directly from dairy products for instance yoghurt.

Conclusions

With the assay we were able to specifically detect lactobacilli strains down to 10^5 cfu ml^{-1} directly from yoghurt, which is a sufficient detection limit as commercial products usually containing 10^6 to 10^{12} cfu ml^{-1} of probiotic strains.

Significance and Impact of the Study

The real-time PCR assay developed here might become a convenient tool enabling an accurate, fast and sensitive detection of probiotic lactobacilli commercially used in food.

2.2 Timely Approaches to Identify Probiotic Species of the Genus *Lactobacillus*

2.2.1 Contribution to this Paper

I designed, structured and prepared the manuscript. In addition, I discussed and interpreted the results. I prepared the final manuscript version after discussing it with all the authors mentioned on the manuscript. WV drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript. LHW drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript and in structural arrangement of the paragraphs. SG drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript and in adjusting the text.
All authors read and approved the final manuscript.

2.2.2 Publication

Herbel, SR, Vahjen, W, Wieler, LH, Guenther, S (2013). Timely approaches to identify probiotic species of the genus *Lactobacillus*. Gut Pathog 5:27.

http://dx.doi.org/10.1186/1757-4749-5-27

2.2.3 Summary

Over the past decades the use of probiotics in food has increased largely due to the manufacturer's interest in placing "healthy" food on the market based on the consumer's ambitions to live healthy. Due to this trend, the usage of probiotic bacteria such as lactobacilli or bifidobacteria is increasing steadily. Thus, probiotics are widely used as starter cultures in dairy products such as cheese or yoghurts and in addition they are also utilized in non-dairy products such as fermented vegetables, fermented meat and pharmaceuticals, thereby, covering a large variety of products.

To assure quality management, several pheno-, physico- and genotyping methods have been established to unambiguously identify probiotic lactobacilli. These methods are often specific enough to identify the probiotic strains at genus and species levels. However, the probiotic ability is often strain dependent and it is impossible to distinguish strains by basic microbiological methods.

Therefore, this review aims to critically summarize and evaluate conventional identification methods for the genus *Lactobacillus*, complemented by techniques that are currently being developed.

2.3 TaqMan® Probes for the Identification and Quantification of Lactobacilli

2.3.1 Contribution to this Paper

I designed, structured and prepared the manuscript and the experiments described in the publication. In addition, I discussed and interpreted the results. I was preparing the final manuscript version after discussing it with all the authors mentioned on the manuscript. MvN-R was responsible for the concept of the project and he drafted and revised the manuscript critically. MK was responsible for the concept of the project and he drafted and revised the manuscript critically. JM designed, structured and prepared the experiments regarding the identification of probiotic species by MALDI-TOF MS. In addition, he drafted and revised the manuscript critically for important intellectual content within MALDI-TOF MS paragraph and has given final approval of the version to be published. LHW drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript, in structural arrangement of the paragraphs and he was responsible for the concept of the project. SG drafted and revised the manuscript critically for important intellectual content and has given final approval of the version to be published. In addition, he took part in writing parts of the manuscript, in adjusting the text and he was responsible for the concept of the project.
All authors read and approved the final manuscript.

2.3.2 Publication

Herbel, SR, Von Nickisch-Rosenegk, MV, Kuhn, M, Murugaiyan, J, Wieler, LH, Guenther, S (2014). Specific TaqMan® Probes for the Identification and Quantification of Lactobacilli in Pharmaceuticals. J Prob Health 2(1): 1000115, 1-6.

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2.3.3 Summary

Several probiotic products containing species of the genus *Lactobacillus* are available on the market. In general, these are fermented dairy products such as yoghurts and vegetables. Additionally, probiotic bacteria are used in pharmaceuticals, which are also believed to have beneficial effects on human health. Common pharmaceutical application forms to deliver probiotics are tablets, drops or granulate formulations for oral administration. They should contain sufficient numbers of viable probiotics to ensure active health benefits. Despite the successful commercialization of lactobacilli, their traditional species identification methods are time-consuming and labor-intensive and do not allow quantification of the species.

Therefore, the objective of the present work was to develop a culture independent, rapid and quantification method for two commercially important species of the genus *Lactobacillus* (*L. acidophilus* and *L. reuteri*). We used a TaqMan® real-time PCR assay based on the *Gro*EL heat shock protein region. Therefore, universal lactobacilli primers and species-specific TaqMan® primers have been developed. The assay allowed an unambiguous species-specific detection of *L. acidophilus* and *L. reuteri* from bacterial cultures as well as directly from tablets.

Using this assay, we were able to detect lactobacilli strains to a level of 10^4 cfu/ml, which is a sufficient detection limit as commercial pharmaceuticals usually contain 10^8–10^{10} cfu/tablet of probiotic strains.

3. Discussion

Probiotics are believed to promote beneficial influences in the GI tract, thereby influencing the health and well-being of humans and animals. For instance, they are believed to produce antimicrobial compounds, reduce luminal pH, exclude pathogenic bacteria, and influence epithelial cells and microbiota composition [68].

In animal feeding, the usage of probiotics has steadily increased due to the increased European Union restrictions in terms of using antibiotics for growth promotion and preventing diseases. Species of the genus *Bacillus* are more prominently used as spores, whereas lactobacilli or bifidobacteria are less frequently utilized in feed due to their reduced ability of being implemented into feed. Naturally, not being able to implement a diverse group such as lactobacilli represents a huge disadvantage.

Thus, even from a diagnostic perspective, a simple validated method for the identification and quantification approaches of different probiotic species within a short time has not yet been established. It is astonishing that an easy to handle technique is still missing. Alternatively, they were able to identify species, yet unable to quantify them, although next to species identity quantification holds predominant importance.

After all, a clear definition had been published by FAO/WHO, pooling the most important aspects such as viability of the cells, a need to have health beneficial effects "when consumed in adequate amounts" [23]. Despite this serious shortcoming, FAO/WHO's definition of probiotics continues to be cited in almost every publication on probiotics. Nevertheless, in nearly every publication, the most important aspects of the original definition are missing: "[…] as part of food[1] […]" and footnote "[1]Water is included as a food." [23]. Despite the shortcomings of FAO/WHO in 2001, no other definition of probiotics have been published so far [23].

Several different probiotic formulations are currently available, including pharmaceuticals and products, which are not part of food such as probiotic bricks or tampons containing probiotic bacteria [145, 146]. Even techniques have been adapted to produce tablets containing freeze-dried or encapsulated probiotic cells, which are still viable, albeit devoid of water or culture media [147]. These examples

of products demand the need to establish an updated globally accepted definition of probiotic bacteria or its cell/DNA material exerting health-supporting effects.
Another serious issue arises when experts discuss the species-/strain-specific dosages needed to exert health benefits. Naturally, this or upcoming issues might be the reason why FAO/WHO definition has not yet been updated. However, a revision of the FAO/WHO definition of 2001 appears unavoidable. To close this gap, the following represents a possible updated definition for probiotics to be in line with any products containing probiotics:

Probiotics are live bacteria or any cell material of microorganisms, which offer health beneficial effects to the host if each strain or strain compartment is administered in adequate amounts and used in dairy and non-dairy products of functional foods, biotherpeutics, probiotic beverages or non-food products.

While this might be an option as a new definition, some parameters are unable to be analyzed by known techniques such as the amount of cell material or cell compartments as well as pieces of the nucleotide sequence, which were shown to have an effect. Currently, it is not possible to test the effectiveness and plausibility of the used compartments due to many parameters, which are influencing microbiota in the GI tract. A further issue is that these effect performers are not easy to separate and cannot be administered in a single.
Thus, it is not astonishing that experts advise morphological and physiological analysis as the first methods to use for the identification of probiotic bacteria, as opposed to molecular biological ones [23]. DNA-based analysis such as genus- or species-specific PCR or RAPD would be much faster and with fewer obstacles in terms of reliability. Instead of this, morphology screening might fail due to different morphotypes of appearances caused by variable environmental conditions [41, 148].

3.1 Health Beneficial Effects, Health Claims & Dosage

Several publications are available that deal with the health beneficial effects of probiotic bacteria [149, 150]. Almost every definition published assumes probiotics to influence and promote health. The FAO/WHO definition of 2001 mentions that

"[…] confer a health benefit on the host […]" [23]. Nonetheless, most available studies focus on patients who are not included or considered in the official definition of probiotics. How has it been possible that scientists ignore sentences within FAO/WHO guidelines containing the information that [23]:

- probiotic bacteria should be used in an adequate amount;
- have to be viable;
- must be part of food, which contains water as a food; and
- that biotherapeutics that are not part of food are excluded from this definition.

It is perhaps worse that probiotics themselves are offering these effects as a stand alone "therapeutic" to meet all needs required to achieve a healthy life. More than hundred years ago, Ilya Illitch Metchnikoff published that good bacteria and food manipulate the microbes within the human gut system [15]. Thus, food, which is implemented in the FAO/WHO definition, has an effect on the intestine and thereby on microflora and probiotics being served within food. Indeed, not only does food affect the intestine; moreover, other important factors such as stress or indisposition affect the "supra-organism", as it was called by Joshua Lederberg [6].

In fact, probiotic bacteria have to be tested in healthy people to analyze their positive effects, if they indeed offer them. In clinical animal trials, the same strains have to be analyzed in "patients" with one or more diseases, while seemingly probiotic infections have to be analyzed using varying amounts or mixtures of bacteria. This may reduce the risk of human infections caused by probiotics, which are used to pretreat or treat patients.

Finally, after investigating starter and added cultures regarding their beneficial power and safety, this significant data can be used within patenting a product. Additional testing *in vitro* and *in vivo* is needed to evaluate probiotic effects for marketing proposes. Even then, it seems impossible nowadays to use data of beneficial effects within health claim advertisements to promote a product's beneficial effect, for instance, in producing bacteriocines.

For this, health claims were established in 2006 to regulate consumers' protection and an effective functioning internal market and fair competition between companies within the European Union (EU) [24, 151]. Unfortunately, the EU neglected to establish a strict catalog of regulations concerning methods and studies needed to

introduce a probiotic product in the European market. Executives focused more on protecting companies than starting to define guidelines to improve standards regarding food ingredients and thereby regulating the usage of probiotics. As an example, the dosage of probiotic bacteria needed to improve health or well-being has not been fixed by the EFSA or EU. Due to missing declarations concerning dosage, even recommended amounts of probiotics are difficult to standardize in international and national authorities worldwide (Table 3) [152]. Therefore, it is unlikely that an overall dosage for all LAB, bifidobacteria and other microorganisms being used as probiotics can be established.

Table 3 Recommended probiotic viability in probiotic foods [152].

Viability Requirements (Min. cfu/ml)	Recommending Agencies	References
10^7 *Lactobacillus acidophilus*	International Dairy Federation	[153]
10^6 Bifidobacteria	International Dairy Federation	[153]
10^6 Lactic cultures	Australian Food Standards Code	[154]
10^8 Lactic acid bacteria	National Yoghurt Association	[155]
10^6 Bifidobacteria	Swiss Food Regulation	[156]
10^6 Bifidobacteria	Fermented Milk and Lactic Acid Beverages Association	[155]
10^7 Lactic acid bacteria	Spanish Yoghurt Quality Standards	[157]

A vast amount of data, studies and other testing is needed to define a quantity necessary to exert beneficial effects. However, interactions between different species of probiotic cells play a role in exerting benefits. Some species have been shown as being triggered regarding their effects when being used in a mixture [79]. Thus, while statements regarding dosage would be important, they would not be enforceable within national or international authorities. Otherwise, each strain and mixture of strains has to be put in a dosage concept.

The EFSA is solely focusing on reliable studies performed on probiotic bacteria. As an example, two opinions of EFSA deal with the effects of probiotic bacteria, which had been confirmed in scientific studies. One of these opinions affirms the lactose reducing effects of *L. delbrueckii* subsp. *bulgaricus* and *St. thermophilus*, if bacteria are used as viable cells in fermented milk products [158]. In another study, *L. casei* DN-114 001 used in Actimel® (Danone®) was evaluated by the EFSA in terms of its ability to reduce *C. difficile* toxins and the risk of acute diarrhea in patients receiving antibiotics [159]. The second opinion was published by EFSA following an application of Danone Produits Frais France® via the Competent Authority of France, the Panel on Dietetic Products, Nutrition and Allergies to deliver an opinion based on scientific data regarding the health claims of the fermented milk drink Actimel® [159]. Within this application, Danone Products Frais France® mentioned several human and animal trial publications to confirm *C. difficile* toxin and diarrhea reducing abilities. However, panel members of the EFSA rejected the application by Danone Produits Frais France® due to insufficient results of *L. casei* reducing diarrhea caused by *C. difficile* [159].

It is surprising that these applications are voluntary and that it is not dictated to offer results of preliminary tests selected by the companies when introducing a new product. Here, similar to the pharmaceutical industry, the food industry should be responsible for assuring the advertised effects. If effects are confirmed by a company's lab, independent laboratories and EFSA manufacturers, they should be permitted to advertise the shown effects of their products.

3.2 Selection and Identification

The viability of bacteria is often affected by stress conditions during manufacturing processes. To avoid the loss of cell viability during the harsh manufacturing conditions, the strains were previously exposed to sublethal environmental conditions such as increased levels of acidity or salt, heat, freezing, dry or oxygen conditions [92, 130]. For instance, such testing can be performed in different genetic machines invented and patented by Mutzel & Marliere (2004, U.S. Patent No. 6,686,194) [160]. Within one approach, bacteria can be exposed up to three different parameters (temperature, medium conditions [salt, acid, etc.] and oxygen/carbon dioxide) to select most robust cells of a species. Using genetic machines adaptation process is less expensive and more rapid, as opposed to testing each parameter individually.

After choosing the most stress resistant species, several techniques are available to identify members of the genus *Lactobacillus*. However, which method is chosen depends on the reliability, usability and coping ability.

First, identification approaches are used in describing the strains after selecting them for product usage. Methods are used to understand and describe a strain's abilities. Accordingly, time-consuming and labor-intensive physiological testings such as API 50 or BIOLOG are used to screen fermentation and temperature capabilities. In addition, these testings are usable for screening components needed for growth medium preparation.

If tested parameters are convincing for later usage in manufacturing approaches, WGS will follow first growth tests. Nowadays, WGS is less expensive to screen genomes of bacteria concerning physiological, stress abilities and possibly pathogenic features. By knowing its threatening or infectious abilities from sequence data, costly in vitro or in vivo studies are not needed in developing "new" strains. Furthermore, WGS offers a vast amount of data that is usable to develop sequence-based identification methods (16S/23S rRNA PCR, PCR-DGGE, RAPD, SSCP, MLST, SSR, real-time PCR, etc.) for species detection. Later on, these methods are applicable for rapid and reliable identification approaches of species used in diverse products.

Utilizing protein-based techniques such as FTIR or MALDI-TOF MS proteins are analyzable, which may have health beneficial effects in humans or animals

influencing mucus layer or epithelial cells. This data could be easily deposited in global databases for later uniform diagnostic purpose.

Although all these described methods are time-consuming and labor-intensive, they are needed to describe the abilities of a strain before utilizing it in product consumption.

A second reason for species identification is the importance of assuring a product's quality standards of dairy and non-dairy products containing probiotics. In this case, timely aspects are an important factor and should not exceed the duration of a working day (< 8 h). Accordingly, culture dependent identification tools based on physiological testing are not feasible. It has been shown that other methods such as DGGE, RAPD, SSCP or SSR are helpful to identify species in a culture independent way. However, some of them require additional analysis approaches including 16S rRNA, RFLP or MLST analysis to confirm detection results. Thus, these approaches are not practicable for daily diagnostics.

There is clearly a need for establishing a rapid, reliable, sensitive and validated method for species-specific identification, including a quantification approach. Within the project, different target genes were tested to identify probiotic species by real-time PCR. In addition, a relatively quick DNA extraction method (within 3 h, modified from Lick et al. [1996]) has been established to isolate DNA from any product containing probiotics [161]. An appropriate method was necessary to extract DNA from fatty milk products without much loss of DNA, even though a minimal amount of DNA gets lost during a phenol-chloroform-isoamyl-alcohol step. However, it has been shown that real-time PCR analysis of DNA extracts attained without cultivation or isolation of the species is workable, reliable and repeatable. Species-specific primers have been established for nine species (*L. acidophilus*, *L. brevis*, *L. delbrueckii* subsp. *bulgaricus*, *L. fermentum*, *L. jensenii*, *L. johnsonii*, *L. helveticus*, *L. reuteri*, *L. salivarius*) of the genus *Lactobacillus* and two species of the genus *Bifidobacterium* (*B. animalis* subsp. *lactis, B. bifidum*), which are often used in food, biotherapeutical formulations or non-food products. Using ten-fold dilutions of an overnight culture of a reference strain (10^8 to 10^3 cfu/ml) and DNA obtained from e.g. yoghurt with real-time PCR approaches, an identification and quantification of detectable species is possible. This reflects a great advantage of the assay and for rapid diagnostic approaches, namely to quantify bacteria

estimating their amount utilized in a product in terms of being in line with FAO/WHO guidelines.

However, even after establishing a good working DNA isolation technique that is useful for different food matrices, DNA interfering events during real-time PCR had to be managed to obtain reliable and analyzable results. For instance, melting curve analysis is a second indicator in real-time PCR analysis to assure correct species amplification. Under fixed annealing conditions, the peak level is fixed to a priory asserted degree level. Later on, by utilizing dilutions of an overnight culture of a strain collection strain and DNA mixtures obtained from Activia® yoghurt (Danone®), the melting curve showed differences of one degree Celsius between specific yoghurt and strain collection amplificates. In following real-time PCR testings using an Activia® (Danone®) DNA mixture and artificial DNA mixtures of the three species used in the yoghurt (*St. thermophilus*, *B. animalis* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*), the same melting curve behavior was demonstrated. In DNA mixtures of two of the three species, the melting curve shifting was less developed than in the samples containing three species in total. Heteroduplex complexes might cause melting curve shifting with a decreased melting curve peak of one degree Celsius difference in comparison to homoduplexes of the ten-fold dilution samples [162, 163]. Naturally, this might be a pitfall within real-time PCR analysis, albeit validatable by prior single strain and mixture DNA testing utilizing same specific primers as for diagnostic approaches. This enables a correct identification and quantification, although melting curve analysis may differ. In addition, DNA sequencing confirmed the correct amplification of the species of interest.

Furthermore, a much faster identification approach has been established by using MALDI-TOF MS analysis. Probiotic species used in tablets, capsule and granulate formulations have been identified within half an hour. However, quantification is not possible. Thus, if species are established in the MALDI-TOF MS database, the bacteria of choice and even contaminants or spoilage bacteria might be detectable within a short time. This approach will be an advantage for the rapid testing of daily charges to assure its safety and quality. Secondary, it is an advantage for scientists and for diagnostic laboratories to equip MALDI-TOF MS databases by introducing additional in-house protein profiles of microorganisms, fungi, plants or animals.

Accordingly, the MALDI-TOF MS database can be deployed in a versatile and convincing way.

Nevertheless, species such as *L. casei* and *L. paracasei* were not distinguishable by real-time PCR. Even species-specific amplification of one of these species failed, caused by unrelated amplification of other species of the genus *Lactobacillus* due to same or similar DNA nucleotide sequences. In 2010, Dr. Ouwehand (DuPont^{TM}) stated in a personal message that "*L. casei* Shirota is actually a *L. paracasei*" [164]. Within the strain catalogue of Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), both species are certainly offered (Table 4). Regarding my request about differentiating these two species, Dr. Pukall (DSMZ) stated in a personal message that "*L. casei, L. paracasei* subsp. *paracasei* and *L. paracasei* subsp. *tolerans* are discriminable using ribotyping and fingerprint analysis" [165].

Until now, it is not possible to distinguish between *L. casei* and *L. paracasei* using real-time PCR analysis. In addition, identification by MALDI-TOF MS still causes misidentification events, assigning them as being *L. zeae* or *L. paracasei* caused by a single data set of *L. casei* being available in the BioTyper database (Bruker, USA) [166, 167].

Table 4 DSMZ strains of *L. casei* / *L. paracasei* (Last update: July 21st, 2014).

Strain	**DSMZ-Catalogue-No.**
L. casei	DSM 20011^{T}
L. paracasei subsp. *paracasei*	DSM 20006
L. paracasei subsp. *paracasei*	DSM 20008
L. paracasei subsp. *paracasei*	DSM 20020
L. paracasei subsp. *paracasei*	DSM 20207
L. paracasei subsp. *paracasei*	DSM 20244
L. paracasei subsp. *paracasei*	DSM 20312
L. paracasei subsp. *paracasei*	DSM 2649
L. paracasei subsp. *paracasei*	DSM 46331
L. paracasei subsp. *paracasei*	DSM 4905
L. paracasei subsp. *paracasei*	DSM 5622^{T}
L. paracasei subsp. *paracasei*	DSM 8741
L. paracasei subsp. *paracasei*	DSM 8742
L. paracasei subsp. *tolerans*	DSM 20012
L. paracasei subsp. *tolerans*	DSM 20258^{T}

3.3 Future Trends

Following the publication of FAO/WHO guidelines in 2001 and the definition of probiotic bacteria, several things have changed [23]. Thirteen years ago, probiotics were served in food that contains water or live microorganisms being embedded in powder milk [23]. Nowadays, probiotic bacteria are more extensively used in food products such as milk products (yoghurt, cheese [168]), as well as being used in meat [169] and vegetable fermentation [142] and within the fermentation process of wine [170] and malt whiskey [171]. Moreover, health beneficial bacteria are even used in non-food products, such as probiotic straws [145]. Herein, bacteria are delivered in an oil drop to guarantee their viability.

Even new manufacturing techniques have been established and probiotics have been successfully included in cereals, chocolate bars and ice cream, although these products are far from being stored at an optimal growth temperature for probiotics (30° C and pH value of 7.0) [172, 173]. The important criterion is that bacteria should not proliferate, although they are still viable within these functional foods as a result of encapsulation or freeze-drying techniques used to protect bacteria in harsh conditions [147, 174]. Perhaps in the future, lactobacilli will be genetically modified to form spores by using essential genes of *Sporolactobacillus* sp., yet still with the ability to ferment lactose. Somehow, it would be a step back to its ancestor *Bacillus*, although more than a step forward in implementing it as spores in any product.

In the coming years, increasingly more different matrices in the area of functional food and non-food products containing probiotics will arise. However, no clear guideline within the EU that deals with the health beneficial effects or health claim testing formalities is available at present. These are needed today and in the future due to the increasing array of products, “new” strains and refaced and updated manufacturing processes. One such regulation should be that companies are more obliged to report successful testing of the strains implemented in food products than in the present scenario. Within establishing the process of new product formulations, diagnostic tools such as real-time PCR approaches can be invented, based upon the company’s own interest to reach the devised quality standards of their products. Naturally, innovations regarding new products will initially decrease, caused by the rising costs for testing and patenting; however, consumers’ safety, quality standards and the effectiveness of the used species will ultimately increase.

3.4 Concluding Remarks

The aim of the present study was to establish a species-specific real-time PCR technique to identify and quantify different probiotic species of the genus *Lactobacillus* in food samples. This genus was chosen because many lactobacilli are widely used for fermentation approaches. However, for diagnostic reasons and to analyze quality standards, an adequate method including quantification has been hitherto missing. The determination of the used species has often been carried out using time-consuming, labor-intensive and costly approaches to assure strain's identity. Indeed, up to 96 hours were needed to identify species, excluding the quantification approach.

As previously shown in molecular-based species-specificity studies, the *Gro*EL (*hsp*60) target region has been successfully adapted to our real-time PCR assay, enabling species detection and the quantification of probiotic lactobacilli [143]. Besides this, the culture-independent DNA isolation method directly from the product origin has been cut in time to afford real-time PCR results within seven hours of work. Furthermore, the applicability of this technique in different matrices (yoghurt, milk drinks, tablets) has been demonstrated. Unfortunately, it was not possible to establish a TaqMan® labeled multiplex real-time PCR assay presumably caused by primer-primer interactions. This would have increased the usability and reduced costs in long time usage in daily diagnostics. Nevertheless, within singleplex TaqMan® real-time PCR, the species *L. reuteri* and its used amount were ascertainable in tablets.

In particular, species used in products have been isolated and identified using physiological, protein-based and molecular biological methods. Thus, viability within yoghurt, tablets or capsules has been successfully demonstrated.

The present study has demonstrated that the real-time PCR approaches are time saving, cost effective, validatable and easy to handle for daily batch diagnostics of probiotic products.

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Summary

Probiotics are believed to promote beneficial influences on the functions of the gastrointestinal tract and thereby on health. Therefore, they are often used in dairy and non-dairy products for human nutrition, including running the fermentation process. Moreover, the feeding of animals with probiotics has also increased, particularly since the European Union approved a prohibition to use antibiotics for growth promotion.

Since then, classic diagnostic methods have been used for the identification of species of the genera *Lactobacillus* and *Bifidobacterium* including phenotypic comparison with reference strains, physiological testings by API 50 CHL stripes (BioMérieux) and polymerase chain reaction (PCR). Different selective media and growth conditions are used to isolate probiotic species from food, resulting in time-consuming, labor-intensive diagnostic approaches. Furthermore, phenotypic characterization and species differentiation are error-prone. Nonetheless, the quantification approach of the used species is still missing, although besides safety, efficacy and viability, its amount also plays a significant role in exerting beneficial effects. Even FAO/WHO guidelines (2001) mention that probiotic bacteria are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [23]. However, this had not led to establishing a molecular-based rapid working tool to conduct diagnostics of probiotic bacteria in a working day.

Given that probiotic action depends on the quality and quantity of the probiotic strains, a conventional PCR detection method is not feasible, as it does not allow any possibility to quantify the species of interest. As it turns out, several other techniques did not work to either detect or quantify.

Thus, a real-time PCR method has been established within this thesis, complying with the requirements to identify and quantify probiotic species in food without a prior cultivation step. Indeed, a scientific method like this has been long overdue.

The screening of different target-sequences for a species-specific identification of probiotic strains ruled out classic targets such as 23s-5s rRNA, due to a lacking species-specificity by multiple amplifications of different species. Thus, other targets such as the heat shock proteins (*Gro*EL) were chosen for the specific identification of different species of the genus *Lactobacillus*. The ATPase subunit of the ATP-

dependent *clp*C gene was selected for the species-specific identification of members of the genus *Bifidobacterium*.

The validation of the real-time PCR primers has been successfully achieved using DNA isolates from several products. Herein, DNA isolated from yoghurt, tablet or granulate origin with probiotic bacteria has been used for specificity and quantification tests.

In addition, species-specific TaqMan® real-time PCR primers have been successfully established for four different species of the genus *Lactobacillus* (*L. acidophilus*, *L. brevis*, *L. helveticus*, *L. reuteri*). Unfortunately, it was not possible to combine these four species-specific primers and the universal primer pairs within one multiplex TaqMan® real-time PCR. Moreover, even a combination of two TaqMan®-labeled primers failed. However, it was possible to demonstrate an accurate and reliable detection and quantification of *L. reuteri* in Reuflor® tablets, respectively.

The main advantage of a real-time PCR method based on the same annealing conditions of different primer pairs is a rapid and species-specific detection, as well as the identification and quantification of different probiotic species within one single real-time PCR run within seven hours. Thus, this enables a fast and reliable diagnostic of probiotic species used in food and feed samples.

Zusammenfassung

Man vermutet, dass probiotische Bakterien einen nützlichen Einfluss auf die Vorgänge im Darmtrakt haben und somit auch gesundheitsförderlich sind. Aufgrund ihrer Eigenschaft, den Fermentationsprozess in beispielsweise Milchprodukten einzuleiten, werden sie häufig in Produkten des täglichen und nichttäglichen Gebrauchs eingesetzt. Außerdem hat der Einsatz von Probiotika in der Tierernährung zugenommen, besonders seit die Europäische Union einen wachstumsfördernden, flächendeckenden Einsatz von Antibiotika verboten hat.

Seitdem werden Spezies der Genera *Lactobacillus* und *Bifidobacterium* mit Hilfe klassischer Methoden identifiziert; hierzu gehören der phänotypische Vergleich mit Referenzstämmen, physiologische Testungen mit API 50 CHL Streifen (BioMérieux) und die Identifizierung mit Hilfe der Polymerase Kettenreaktion (PCR). Verschiedene Selektionsmedien werden zur Isolation probiotischer Spezien aus Lebensmitteln verwendet, die jedoch zeit- und arbeitsintensive Diagnostikmethoden erfordern. Die phänotypische Charakterisierung und die Spezies-Differenzierung sind sehr fehlerbehaftet. Hinzu kommt, dass es noch immer keine Quantifizierungsmöglichkeit gibt. Von Bedeutung sind neben der Unbedenklichkeit der eingesetzten Bakterienstämme auch die Effektivität und die Lebensfähigkeit der eingesetzten Bakterien. In den FAO/WHO-Richtlinien von 2001 steht, dass probiotische Bakterien “lebende Mikroorganismen sind, die, wenn sie in ausreichender Menge eingesetzt werden, eine gesundheitsfördernde Eigenschaft auf den Konsumenten haben”. Selbst diese Richtlinien haben nicht zur Etablierung einer schnellen, wissenschaftlichen und molekularbiologischen Methode geführt, um die Diagnostik probiotischer Bakterien innerhalb eines Arbeitstages durchführen zu können.

Da die Wirkungsweise auf der Qualität und Quantität des vorhandenen probiotischen Bakterienstammes beruht, kann die konventionelle PCR Detektionsmethode nicht angewandt werden, da diese keinerlei Möglichkeit bietet, die zu detektierende Spezies auch zu quantifizieren.

In dieser Arbeit wird eine real-time PCR Methode etabliert, um den Anforderungen der Identifizierung und der Quantifizierung gerecht zu werden. Eine solche Methode war längst überfällig.

Es wurden verschiedene Target-Sequenzen zur spezies-spezifischen Identifizierung probiotischer Bakterien getestet und klassische Sequenzen wie die 23s-5s rRNA aufgrund fehlender Spezies-Spezifität durch Mehrfachamplifikationen verschiedener Spezies verworfen. Andere Targetgene wie beispielsweise das Hitzeschockprotein (*Gro*EL) wurden zur Identifizierung verschiedener Spezies des Genus *Lactobacillus* ausgewählt. Die ATPase Untereinheit des ATP-abhängigen *clp*C-Gens wurde genutzt, um Spezies des Genus *Bifidobacterium* spezies-spezifisch nachweisen zu können.
Zur Validierung dieser real-time PCR Primer wurden DNA-Isolate aus verschiedenen Produkten verwendet, wie beispielsweise DNA-Isolate aus Joghurt, Tabletten und Granulat. Sie wurden für die Spezifitäts- und Quantifizierungstests eingesetzt.
In einem weiteren Ansatz wurden spezies-spezifische TaqMan® real-time PCR Primer für vier verschiedene Spezies des Genus *Lactobacillus* (*L. acidophilus*, *L. brevis*, *L. helveticus*, *L. reuteri*) erfolgreich etabliert. Es war nicht möglich, diese vier verschiedenen spezies-spezifischen Primer sowie die Universalprimerpaare erfolgreich in einer multiplex TaqMan® real-time PCR zu kombinieren. Sogar die Kombination von nur zwei TaqMan®-gelabelten Primern misslang. Es konnte aber gezeigt werden, dass eine akkurate und verlässliche Detektion und Quantifizierung von *L. reuteri* in Reuflor® Tabletten möglich war.
Der eigentliche Vorteil der real-time PCR basiert auf der schnellen spezies-spezifischen Detektion, Identifikation und Quantifizierung verschiedener probiotischer Spezies während eines einzigen real-time PCR-Laufs innerhalb von sieben Stunden. Das ermöglicht eine schnelle und eindeutige Diagnostik probiotischer Spezies in Lebens- und Futtermittelproben.

Parts of the thesis had been presented in an oral presentation on the "International Conference and Exhibition on Probiotics 2012", OMICS Group, San Antonio, USA (19.-21.11.2012). The presentation had been ranked as the best talk within the Young Scientist Challenge Trophy Competition.

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For ranking in First Place in Young Scientist Challenge Trophy Competition entitled DETECTION, IDENTIFICATION & QUANTIFICATION OF DIFFERENT PROBIOTIC STRAINS IN FOOD BY USING MULTIPLEX PCR METHOD

Awarded: November 21, 2012

G. Sainubabu
Gedela S. Babu
Director, OMICS Group Inc., USA

S. K. Dash
South Dakota State University, USA
On behalf of Organizing Committee

Curriculum Vitae

For reasons of data protection,
the curriculum vitae is not included in the online version.

Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit ohne die unzulässige Hilfe Dritter und ohne die Verwendung anderer als der angegebenen Hilfsmittel angefertigt habe.
Diese Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Berlin, den 22. Juli 2014

Stefan Roland Herbel