Gut Pathogens

REVIEW

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Timely approaches to identify probiotic species of the genus *Lactobacillus*

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Abstract

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, health benefits of products containing probiotic strains such as lactobacilli are promoted and probiotic strains have been established in many different products with their numbers increasing steadily. Probiotics are 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.

Introduction

Members of the genus *Lactobacillus* are Gram positive, acid tolerant, facultative anaerobic and fermentative bacteria with low $G + C$ content belonging to the phylum Firmicutes [1]. They are common in food related habitats such as wine, milk, meat, fruits, vegetables and cereal grains and are often used as starter cultures for food fermentation processes [2]. Additionally, some members of the genus *Lactobacillus* are naturally associated with mucosal surfaces, residing in parts of the intestinal tract, vaginal and oral cavity of humans and animals [3]. Lactobacilli have been used for millennia for the preservation of food, e.g. cured meat such as salami or pickeled vegetables such as sauerkraut and olives. They are also very common as starter and adjunct cultures of dairy products such as yoghurt and cheese [4]. Food which is claimed to have a beneficial effect on the consumer's health by using microbial dietary ingredients are known as functional, designer or fortified food containing probiotics [5].

Over the last decades a wide range of these functional products containing probiotics have been made available in the market which is fostered by the current trend of consuming healthy food in order to prevent illness [6]. Besides bifidobacteria, lactobacilli are currently among the most important probiotics and species like *Lactobacillus casei* are widely used in food supplements and lactic beverages such as Yakult® (Yakult, Germany) or Actimel® (Danone, Germany) [7].

The word "probiotic" is a composite of the Latin preposition pro ("for") and the Greek adjective of the noun βίος (bios, "life")" [8]. Therefore, the viability of probiotic bacteria within products is crucial for the beneficial effects they intend to offer to the consumer's health. For example in order to offer beneficial effects to the host, 10^6 to 10^8 colony-forming units (cfu) per ml are needed as viable bacteria till the end of storage time [9,10]. Nevertheless, several studies showed low survival rates of utilized probiotic strains within storage time of products [9].

For quality management reasons and in order to adhere to the European Health Claims Regulations (EC, No 1924/2006 of the European Parliament and of the Council of 20th December 2006) fast and reliable tools are needed to identify and quantify probiotic strains used in a product [7,11].

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Despite the large economic impact of lactobacilli most of the assays currently used for their identification are classical microbiological methods which are time consuming and not easy to standardize. Furthermore, phenotypic identification may fail due to misidentification [12]. These basic phenotypic methods include morphology, Gram staining and biochemical tests such as fermentation of carbohydrates or growth at varying temperatures and salt concentrations [12]. Morphology screening for differentiation seems problematic, as it is known that lactobacilli have diverse morphotypes within the same species [13]. Fortunately, for species-specific identification of strains based on physiological properties other modern tools have become available over the last years such as the API system from Biomérieux (France), the Diatabs system (Rosco, Denmark) or the BIOLOG GP MicroPlate System (BIOLOG Inc., USA). Additionally, rapid identification tools based on genomic features of lactobacilli include 16S or 16S-23S rDNA (ITS)-PCR and quantitative real time PCR analysis or proteomic analysis using MALDI-TOF MS [7] (Table 1).

This review aims to evaluate classical microbiological identification methods of the genus *Lactobacillus* complemented by newly developed molecular techniques. As probiotic lactobacilli are successfully used currently, the first chapter of the review will deal with a short overview on probiotic health benefits. In the second part different phenotypic, physicochemical and genotypic methods will be discussed. The detection level in terms of genus, species, subspecies and strain specificity will also be in focus. As probiotic effects are often strain dependent the latter is of utmost importance.

Probiotics

Definition

In 1989 Fuller defined probiotics for animals as 'a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance' [16]. By using this definition he assumed probiotics as live bacteria which have a beneficial effect on the host. Later on Schrezenmeir and de Vrese (2001) amended the definition as 'a preparation of or a product containing viable, defined

Table 1 Experiment duration and detection level for each method

Method	Duration (h[a])	Time in total (h)		Detection at the level of…			Quantification
		Culture-dependent	Culture-independent[b]	Genus	Species	Subspecies	
Morphology	*[c]	~ 48	/[d]	−[e]	−	−	−
FTIR	* + 1 h (analyzation)	~ 49	/	+[f]	+	−	−
MALDI-TOF MS	* + 1 h (analyzation)	~ 49	/	+	+	+	−
API 50 CHL	* + 48 h (incubation)	~ 96	/	+	+	−	−
BIOLOG	* + 25 h (24 h incubation of AN MicroPlate™ + 1 h analyzation)	~ 72	/	+	+	−	−
16S/23S rRNA PCR + sequencing	* + 31 h (4 h DNA isolation + 3 h PCR + 24 h sequencing)	~ 79	~ 31	+	+	−	−
PCR-DGGE	* + 7 h (4 h DNA isolation + 3 h PCR + electrophoresis)	~ 55	~ 7	+	+	−	(+)[g]
RAPD	* + 7 h (4 h DNA isolation + 3 h PCR + electrophoresis)	~ 55	~ 7	+	+	+	−
SSCP	* + 7 h (4 h DNA isolation + 3 h PCR + electrophoresis)	~ 55	~ 7	+	+	−	−
MLST	* + 31 h (4 h DNA isolation + 3 h qPCR + 24 h sequencing)	~ 79	~ 31	+	+	+	−
qPCR	* + 7 h (4 h DNA isolation + 3 h qPCR)	~ 55	~ 7	+	+	−	+
SSR	* + 7 h (4 h DNA isolation + 3 h PCR + electrophoresis)	~ 55	~ 7	+	+	+	−
WGS	* + 4 h DNA isolation + 36 h Sequencing, annotation, etc.)	~ 88	~ 40	+	+	+	−

[a] h, hour(s).
[b] duration exclusive cultivation of the strains (48 h).
[c] *, isolation of the strains by plating on different media (MRS broth [Roth, Germany [14]], LBS agar [Becton Dickinson, USA [15]], COL and CHOC plates [Sarstedt, Germany]).
[d] /, identification of the strains not possible by working culture-independent.
[e] −, detection at the level of… is not possible.
[f] +, detection at the level of… is possible.
[g] (+), a limited quantification is possible using PCR-DGGE.

microorganisms in sufficient numbers, which alter the microbiota (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host' [17]. In 2010 the World Health Organization (WHO) defined probiotic strains as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' [18]. In general, bacteria have to comply with the following selection criteria to be cited as 'probiotics' [19,20]:

– be of human or bovine origin and non-pathogenic,
– sustain integration into food in high cell counts,
– maintain their viability throughout shelf-life of the product,
– be resistant towards bile and acid juice and withstand transition through the GI tract,
– be an antagonist towards pathogenic bacteria in the gut,
– offer health benefits.

Probiotic bacteria offer a wide range of beneficial effects. They are able to decrease the duration of diarrhea, reduce allergic syndromes, deliver various bacteriocins and lower the pH subsequently inhibiting invasion of pathogens such as *Salmonella* spp. or *Escherichia coli* [21].

As mentioned above, probiotics are widely used in fermented food and feed due to their presumed beneficial effects on host's health. However, these health benefits are often strain specific, therefore unambiguous identification to species and strain level is important [22]. This has been found by using strains singly and in combination with other strains resulting in a reduced or suppressed effect of their benefits when used in combination [23]. Thus, each new combination of probiotic strains has to be studied to avoid the use of non-functional probiotic bacteria [23].

Most probiotic organisms currently used in food for humans belong to either the genus *Lactobacillus* or *Bifidobacterium*. Bifidobacteria are Gram positive, non-motile, non-sporulating, anaerobic and hetero-fermentative bacteria with a high G + C content. Members of the genus *Lactobacillus* are also Gram positive, non-motile and non-sporulating; however, they are acid tolerant and facultative anaerobes, homo- or heterofermentative and have a low G + C content.

Other bacteria used as probiotics in human and animal nutrition include *Escherichia coli* strain Nissle 1917, *Lactococcus lactis*, *Streptococcus thermophilus* and *Enterococcus faecium* (Wysong, USA). Additionally, for more than three decades *Bacillus toyonensis* sp. nov. (formerly described as *Bacillus cereus* var *toyoi*, [24]) has been used in animal nutrition as a probiotic due to its spore-forming abilities that withstand thermal processing of animal feed [25]. Fungi such as yeasts of the species *Saccharomyces cerevisiae* and *Saccharomyces boullardii* are also used as probiotics [26].

It is crucial for probiotics to survive the gastric passage from oral uptake to the gastrointestinal tract (GI) having a beneficial effect [27]. Although, dead probiotic cells are believed to offer a positive effect on the GI as well, they lose most of their probiotic effect with the loss of viability [28]. Nevertheless, it has been shown that cell compartments such as peptidoglycan or lipoteichoic acids of *L. rhamnosus* GG have an effect on the immune system. Iliev et al. (2005) demonstrated that even the genomic DNA sequence TTTCGTTT of *L. rhamnosus* GG was able to stimulate both murine and human immune cells [29,30].

Health benefits

Recently, several reviews regarding the potential systemic and GI specific health benefits of probiotics have been published [27,31]. Various probiotic strains are used in pharmaceuticals such as drops or tablets to prevent or treat intestinal diseases by claiming to exploit the antimicrobial activity of some probiotics [32]. Infectious diarrhea, a major problem in both developing and developed countries, is an intestinal disease in which probiotic therapies are utilized [33]. In addition probiotics might also play an important role in human depressive disorders and may influence brain function and behavior [34]. Probiotic effects are restricted to certain strains and are not found broadly within an entire species or genus [35]. Adhesion or aggregation activity is closely related to properties of the bacterial surface layer (S-layer) [36]. The S-layers are self-assembled proteinaceous, planar subunits forming monomolecular-thick crystalline lattices. A few specific functions have been reported for the S-layer such as being a protective coat, a molecular and ion trap and being involved in cell adhesion and surface recognition [36]. These S-layer characteristics are species-specific and are presently not regarded as being genera-specific among the Firmicutes [37].

Probiotic bacteria confer health benefits in diverse ways. Some are able to modify the populations of the gut microbiota by influencing metabolic and nutritional functions of commensal bacteria [38]. Others show indirect and/or direct immune modulating capacities, often by delivering antigens, modulating sensory motor functions, enhancing mucosal barrier functions and/or providing anti-pathogenic effects [38]. Others may prevent metabolic conditions by lowering cholesterol and improving lactose tolerance [39]. Furthermore, probiotics have been reported having positive effects in some gastro intestinal diseases such as inflammatory bowel disease, by being anti-diarrheal and anti-mutagenic [26,40]. Adjuvant effects of probiotics are also used to improve vaccine efficacy [35].

The food industry is promoting these health benefits based on ongoing scientific research. These studies published should help to determine a prophylactic daily dosage to ensure a therapeutic benefit to the consumer [9,31,40]. Many of the strains used like *L. rhamnosus* GG (*Valio* Ltd., Finland), *L. casei* Shirota (Yakult) and *B. animalis* Bb-12 (Chr. Hansen, Denmark) have been studied in detail concerning their beneficial health effects. In case of rotaviral diarrhea, chronic gastrointestinal inflammatory disorders, antibiotic induced diarrhea caused by broad-spectrum antibiotics or diarrhea caused by *Clostridium difficile*, probiotics have been shown to reduce the length and number of episodes [5,41,42]. This is also true for strain *L. casei* DN-114 001 which has been shown to inhibit the interaction of adherent-invasive *E. coli* with intestinal epithelial cells, thereby having a therapeutic effect in Crohn's disease [43].

In controlled human trials, Ciorba et al. (2012) found that fortified yoghurt containing adequate amounts of viable probiotic bacteria does have beneficial effects on human health [38]. Nevertheless, it is worth mentioning that these effects are also well known for the consumption of fermented food such as red wine, tempeh, red yeast and rice as folk medicine in countries such as India, China and Japan [44].

Identification methods for members of the genus *Lactobacillus*
Phenotypic identification
Morphology
The identification of strains or species of the genus *Lactobacillus* solely by colony or cell morphology is impossible, however, these characteristics do provide an initial overview of the bacteria present in a product before identifying them using other phenotypic methods or genotyping [45]. Phenotypic methods used either alone or in combination to support cell morphology screening include cell motility testing, Gram staining, and catalase and oxidase reactions [45].

API 50 CHL
To differentiate bacterial isolates by their physiological properties various tests are available based on fermentation properties of bacteria. The API 50 CHL system from BioMeriéux (Biomérieux, France) can be used to identify probiotic lactobacilli by testing their fermentation capabilities (fermentative and phenotypic profiling) [46]. The system utilizes a wide spectrum of physiological tests, including substrates covering carbohydrates, heterosides, polyalcohols and uronic acids. Assimilatory, oxidative and fermentative pathways are derived from growth and color changes caused by pH changes.

Globally, many diagnostic laboratories rely on this phenotypic characterization method to identify members of the genus *Lactobacillus* in samples from conditions such as vaginosis. By elucidating the strain's specific physiological needs this procedure provides an inside view to assimilatory, oxidative and fermentative pathways within one test run. According to the manufacturer's instructions the results have to be analyzed 48 h after incubation with the APIweb database offered by BioMeriéux (Biomérieux, France) [47].

Reports on the specificity of this method are ambiguous. The most common vaginal bacterial strain, *L. acidophilus*, has been successfully identified by utilizing this method. However, other studies reported limitations of the API 50 CHL system as identical *Lactobacillus acidophilus* strains showed different phenotypic patterns or were nonreactive for all 50 tests included [48]. Boyd et al. (2005) found that one third (33 of 97) of strains identified via API 50 CHL were not specifically identified [49]. A discrepancy of the API 50 CHL results and the known original species was also shown by Nagy et al. (1991) and Alvarez-Olmos et al. (2004) [46,50]. Furthermore, even the APIweb database sometimes caused misidentification or misinterpreted results [49].

A study regarding the isolation of probiotic lactobacilli from fermented traditional food such as kocho (fermented plant powder for bread) and tef flour (whole grain flour) samples in south and south-western Ethiopia compared molecular and phenotypic based methods for the identification of isolated strains and found a discrepancy in the results received from API 50 CHL stripes and Randomly Amplified Polymorphic DNA-PCR (RAPD) cluster analysis [51]. In another study a discrepancy was also detectable when analyzing the phenotypic patterns of members of the *L. casei* group (*L. casei, L. rhamnosus, L. zeae*) with this method [52]. The identification of closely related strains was deemed unacceptable due to the misidentification of a *L. casei* strain as *L. rhamnosus* [52].

In conclusion the API 50 CHL system seems to be appropriate for underlining results based on genomic methods; however, due to the high level of phenotypic variability among lactobacilli this lab-intensive method should not be solely used [49]. In addition, results of API 50 CHL stripes can show acidification processes instead of growth or fermentation processes and even oxygen or a deviation in the density of the bacterial suspension may affect the output. Misidentification and non-interpretable results are clear pitfalls of this method [46,50].

Metabolic activity testing using BIOLOG
BIOLOG AN Microplate® (Biolog, Inc., Hayward, CA, USA) was designed to identify members of the genera *Bifidobacterium, Clostridium, Eubacterium, Fusobacterium, Lactobacillus, Lactococcus, Megasphaera, Pectinatus, Pediococcus, Peptostreptococcus, Propionibacterium* and *Weissella* [53]. The BIOLOG system uses tetrazolium and formazan

deposition as violet indicators for substrate oxidation in bacterial metabolism processes [54]. These tests are performed simultaneously, and result in a metabolic fingerprint of a strain exposed to 95 different carbon sources [53]. Data are collected by BIOLOG Automatic Reading Instrument and analyzed by BIOLOG MicroLogTM software with the connected database (Biolog, Inc., Hayward, CA, USA) in order to identify the tested strain [53]. The software itself has to be optimized to unambiguously identify a particular species.

However, identification of members of the genus *Lactobacillus* might be difficult. For instance, neither amino acids nor their derivatives are used as a carbon source by *L. rossiae* [55]. De Angelis et al. (2007) mentioned that LAB fermentation capacity varies from very few (e.g. *L. sanfranciscensis*) to a broad range of substrates (e.g. *L. plantarum*) being fermented in BIOLOG's testing system [56]. In contrast to this, it is possible to analyze physiological abilities within one species. *L. plantarum* strains differed in the fermentation of glycerol, D-malic acid, D-galacturonic acid, inosine, D-sorbitol and D-ketobutyric acid [57].

Although the BIOLOG identification tool offers a wide scope of physiological tests, currently, an unambiguous identification of strains does not seem to be possible [58]. Nevertheless, it appears to be a useful tool to confirm results based on other phenotypic or genotypic tests and to identify the physiological needs and fermentation potential of a particular strain [57,58].

Physico-chemical identification
Fourier Transformation Infra-Red spectroscopy (FTIR)
Since 1911 infrared spectroscopy has been used to analyze biological samples. Between the 1950s and 1960s the popularity of spectroscopy resulted in the development of many new infrared (IR) light-technologies to distinguish microorganisms. Despite this, the approach lost its popularity due to unsatisfactory results [59]. Developed as a result of computer technology and new statistical analysis techniques, Fourier Transformation Infra-Red spectroscopy (FTIR) now presents a much more efficient tool to identify bacteria [60]. In general, IR light is a widely used technique to analyze molecules by identifying their rotation and spinning spectra within seconds [61]. Fourier Transformation Infra-Red spectroscopy (FTIR) uses polychromatic IR light to analyze the rotation and spinning of components in a bacterial sample after continuously firing certain wavelengths of laser light onto it [62].

For instance, in comparison to physiological methods liquid cultures are easy to handle for the analyzation by FTIR. No prior sample preparation is needed and in addition, any physiological state of a sample can be used for rotation and spinning analysis. The spectra are compared to reference data available in the software (Bruker, Billerica, MA, USA) [63].

FTIR technology enables differentiation of bacteria by studying their cell components, fatty acids, membrane and cellular proteins, polysaccharides and nucleic acids [59]. Isolates from diverse food or feed environments such as identification of starter and non-starter cultures from cheese origin can be analyzed [64]. For instance, *L. kefir* shows different surface properties regarding the structure of the S-layer in comparison to other lactobacilli which is important for elucidating their functional abilities (fermentation properties, etc.) [36]. FTIR spectroscopy even allows the identification of intact encapsulated probiotic cells thermally processed in beads being used in environments such as cereals. Starch or sucrose encapsulated probiotic bacteria are able to be analyzed by species-specific proteins, nucleic acids or components of the membrane [64].

The method is rapid, inexpensive, sensitive and allows high throughput analyses for the identification of bacteria [64]. In contrast to other methods such as morphology screening or phenotypic approaches FTIR spectroscopy enables a differentiation of bacteria at the genus, species and strain level (Table 1). However, there are publications available which report about the limitations of using this technique as a single method for identification [65]. Therefore, other methods should be used to confirm FTIR spectrometry results [65]. For instance, in one study comparison of FTIR results with 16S rRNA sequencing confirmed the spectroscopic findings [64].

Matrix-Assisted Laser Desorption Ionization - Time Of Flight Mass Spectrometry (MALDI-TOF MS)
Each molecule has its own characteristic weight and Matrix-Assisted Laser Desorption Ionization (MALDI) can be used for the characterization of large biomolecules and bacterial proteins with a mass range of 2 kDa and 12 kDa [66]. Astonishingly, whole bacterial cells of overnight cultures can be used for chemotaxonomic classification employing MALDI [67]. This led to a rapid development of MALDI-TOF MS methods for the characterization of targeted or unknown proteins, bacterial RNA and DNA to the level of genus, species, sub-species and strain level (Table 1) [68].

Detecting the protein content of unknown bacteria has to be done by using a matrix of aromatic compounds that are placed and dried on the target before being placed in MALDI-TOF MS aperture [69]. By tearing the matrix sample with a nitrogen laser system (wavelength: 337 nm) molecules are desorbed and ionized in the vacuum [69]. Smaller matrix molecules are heated up by the laser and larger sample molecules are entrained [69,70].

As an example, differentiation of *Lactobacillus casei* and *L. paracasei* is challenging as both species belong

to the *L. casei* group (*L. casei*, *L. paracasei*, *L. zeae*, *L. rhamnosus*) [71]. As these two species cannot be differentiated by conventional phenotypic methods or by MALDI-TOF MS, a combination of methods (e.g. PCR or 16S Amplified Ribosomal DNA Restriction Analysis [ARDRA]) has to be used for correct identification [72]. Additionally, data of a single *L. casei* strain are available in the BioTyper database (Bruker, USA) cause a misidentification of strains of the same species assigning them to *L. zeae* or *L. paracasei* [66,72]. In contrast, MALDI-TOF MS successfully worked in subspecies determination of two *L. delbrueckii* subsp. *bulgaricus* strains [66].

Within minutes the method enables an extended phenotypic identification of lactobacilli, as the spectrum is embedded in the software of the company [68]. Several commercial software packages are available for microbial species identification such as MALDI Biotyper (Bruker, USA), Axima (Shimadzu, USA), SARAMIS (AnagnosTec, Germany) systems (renamed as VITEK MS [Biomérieux, France]), Andromas (Andromas SAS, France) systems and MicrobeLynx (Waters, USA) [29].

MALDI-TOF MS is a rapid and simple tool for the identification of lactobacilli, although the costs accompanying the purchase and running of a MALDI-TOF MS are extremely high [68,73]. Thus, it is increasingly used in diagnostic laboratories solely or in combination with other methods such as 16S rRNA sequencing to differentiate closely related species [73].

Genotypic identification
Sequencing of 16S/23S-5S rRNA
The 16S rRNA presents the most common target region for phylogenetic analysis at the species level, because sequence data of this region can be used for taxonomic classification. PCR products are easily analyzed using species-specific primers of 16S-23S rRNA and gel electrophoresis [74]. Explicit strain identity is managed by additional sequence analysis. This can be done either by Sanger or pyrosequencing (454), by single-molecule real-time (SMRT) sequencing (Pacific Biosciences, USA), ion semiconductor (Ion Torrent sequencing, Life technologies™, Applied Biosystems, USA), sequencing by synthesis (Illumina, USA) or sequencing by ligation (SOLiD sequencing, Life technologies™, Applied Biosystems, USA) [75]. Likewise, sequence data analysis offers an inside view by BLAST (database of the National Center for Biotechnology Information) or Megalign® alignment suite (Lasergene DNAStar, USA) using the ClustalW algorithm [34]. The intergenic spacer regions (ITS) of 16S-23S-5S rRNA are commonly used to identify LAB, especially lactobacilli [76]. Using colony PCR, a crude cell lysate and species-specific primers targeting the 16S rRNA offers rapid identification of lactobacilli within three hours after isolation (duration for cultivation: 48 h) [76]. A precise

assignment of lactobacilli to the level of genus or species is possible utilizing sequences of the 16S-23S-5S rRNA region. Many primers of 16S- or 16S-23S rRNA regions are available discriminating members of the genus *Lactobacillus* at species level using PCR, Denaturing Gradient Gel Electrophoresis (DGGE), RAPD, pulsed-field gel electrophoresis and other methods discussed further on [66,77,78].

However, while 16S/23S-5S rRNA sequencing is useful to identify members of the genus *Lactobacillus* in daily diagnostics, too much time is needed to sequence PCR products and analyze sequence readouts getting reliable results.

Quantitative real time Polymerase Chain Reaction (qPCR)
Quantitative real time Polymerase Chain Reaction (qPCR) is a culture-independent and molecular based method. It enables the discrimination of different species and to quantify the amount of bacteria used in a sample. In real time qPCR analysis it is possible using different PCR techniques to measure the amplification process by genus or species-specific primers [79]. For instance, SYBR® Green, TaqMan® labeled primers or molecular beacons are commonly used qPCR techniques.

SYBR® Green is a DNA-binding fluorescence dye which has an affinity to bind to double-stranded DNA (dsDNA) [80]. In contrast to SYBR® Green TaqMan® labeled primers and the molecular beacon technique are probe-based assays marked with a reporter-quencher system [81]. For species-specific detection of a strain a species-specific TaqMan® labeled primer is designed annealing to a sequence internal flanking universal primers.

Molecular beacon probes form hairpins and are not fluorescent in this non-hybridized state [82]. Thus, using one of these methods detection and quantification of a strain is possible without using further post-PCR analyzes steps, if a strain specific sequence is known.

To enumerate bacteria in complex bacterial communities qPCR allows a quantitative approach [83]. Reverse transcription quantitative real time polymerase chain reaction (rT-qPCR) enables the study of growth abilities and activity of bacteria in food estimating their gene expression [84]. Being rapid and culture independent, qPCR is a highly sensitive, specific and accurate method enabling a simultaneous detection and quantification of bacteria in microbial communities [84].

In comparison to culture-based methods qPCR is more rapid and it is possible to detect minor populations of bacteria within dominant populations [85]. Even non-cultivable species (starter cultures of members of the genus *Lactobacillus* in yoghurt containing *Streptococcus thermophilus*) can be detected and quantified using qPCR and PCR [85].

Both, qPCR or rT-qPCR are inexpensive and suitable for daily routine analysis [85]. As post-amplification manipulations are not necessary the risk of contaminations is limited [85]. Under strict and established PCR conditions, C_T-values and melting curve analysis are tools assuring strain identity. Thus, qPCR are ideal methods for species-specific quantification and identification of bacteria [86].

Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

PCR-DGGE is a molecular based method dealing with the analysis of DNA and does not require prior cell cultivation or separation of individual strains. Microbial communities used in probiotic products are easily evaluated with this method. Different DNA sequences have different melting temperatures due to variations in nucleotide composition. Using Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis (PCR-DGGE) PCR products of the same length can be separated in denaturing gradient gels based on sequence differences. The migration process of the double stranded DNA through the gel stops at its specific melting temperature and separation into single stranded DNA [87]. Then, resulting bands in the gel can be analyzed by comparing them to the control DNA run on the same gel. The intensity of the bands on a DGGE gel is a semiquantitative measure to visualize the dominance of certain strains in the sample over less dominant species [88]. Thus, a limited quantification might also be possible using this approach.

Several publications describe the identification of bacterial communities by PCR-DGGE in cheese [89], sausages [90], wine [91], sourdough [92] and malt whiskey [93]. Many primers are available to amplify sequences of bacteria used in probiotic products, differentiate lactobacilli in GI communities and African and Irish kefir grains.

One drawback to PCR-DGGE is that minor species might not be detectable with this method if they are present in total bacteria populations with less than 1% of the total population [88]. Another drawback is that closely related strains such as *L. casei* / *L. paracasei* might result in equal band sizes in DGGE gel resulting in the misidentification of *L. paracasei* as *L. casei* [94]. In addition, target genes such as *cpn*60 and *rpo*B seem to have a higher discriminative power than 16S rRNA.

However, PCR-DGGE should not be used without additional sequencing of 16S rRNA to assure results [88,95]. In contrast, there might be a lack in designating species by sequencing 16S rRNA PCR products due to high sequence similarity [94]. To avoid false identification a combination of PCR-DGGE and 16S rRNA sequencing of the V3 region might be a potential tool to discriminate species to inter- and subspecies relationships

[94]. Additionally, it is time-consuming to identify single bands [96].

Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR)

Arbitrary primers are adopted in Randomly Amplified Polymorphic DNA-PCR (RAPD-PCR). These short unspecific primers anneal to multiple random target sequences and lead to band "fingerprints" to distinguish different bacteria [97,98]. A high number of samples can be analyzed within a short time [99].

Several publications report heterogeneities of LAB that can be differentiated by using RAPD-PCR. As an example, this technique was successfully applied to distinguish between *L. helveticus*, *L. sake*, *L. plantarum* and *L. delbrueckii* subsp. *bulgaricus* at both an interspecies and intraspecies level [100,101].

A newly developed Ready-To-Go-RAPD kit decreases the time needed to screen of bacterial communities containing necessary primers [102]. This kit enables the user to follow progress of starter culture activities in vegetable fermentation processes similar to sauerkraut [103].

Even for inexperienced users RAPD is easy to perform and cheap [104]. It does not require prior knowledge of specific sequences to characterize and distinguish bacteria at subspecies levels [102,103]. Gosiewski et al. (2012) observed that the Ready-To-Go-RAPD kit did not discriminate between *L. plantarum* strains of human origin, however, small degrees of variations were detectable in *L. plantarum* strains from plant reference strains [102]. Comparing RAPD and PFGE methods, RAPD has a lower degree of differentiation power among strains such as *L. fermentum* and *L. gasseri* than PFGE [102]. Other authors have reported similar findings indicating that RAPD results are less efficient in comparison to the Pulse-Field-Gel-Elecrophoresis (PFGE) technique [105].

Another pitfall of the RAPD procedure is a reported difficulty in obtaining repeatable results [103]. The procedure can be sensitive to variations in sample preparation resulting in variable results between samples of the same species or strain origin. Therefore, this technique should not be used as a stand-alone method [103]. However, it is a useful technique to confirm results of lactobacilli received by PFGE.

Single-strand conformation polymorphism (SSCP)

Frequently, single DNA or RNA strands have a high affinity to form base pairs. However, if no complementary DNA or RNA strand is available, RNA or single stranded DNA form folded conformations with themselves. It is dependent on criteria such as sequence properties and temperature conditions to constitute different conformations. A single mutation in a DNA or RNA strand causes a shift in the single strand modification affecting mobility in gel electrophoresis. If the underlying criteria are

known, it is simple to induce self- or folded conformations by single DNA or RNA strands using their DNA fragments in a temperature gradient gel electrophoresis to identify diverse bacterial communities [106].

The Single-strand conformation polymorphism (SSCP) method is also a basis to analyze 16S rRNA [107]. It is a culture-independent tool evaluating LAB communities in food such as raw milk [108], traditional cheeses [109] and fermented fish products [110]. Variations in the V2-V3 region of 16S rRNA are used to identify strains by comparing their SSCP profiles towards reference strains [65]. Obtaining reliable results is possible when combining Restriction Fragment Length Polymorphism (RFLP) for genus identification with SSCP for species identification [65,108]. Additionally, members of the genus *Lactobacillus* are identifiable by combining sequencing and SSCP analysis.

SSCP is a sensitive and accurate procedure to identify bacteria in different environments if methods such as RFLP or sequencing of V2-V3 region of 16S rRNA are used to assure results on species and strain level [20]. Diagnostics using SSCP are less time-consuming and expensive than establishing species-specific primers for PCR. In addition, it is a DNA sequence-based method which does not need any sequence analysis software.

Multilocus Sequence Typing (MLST)
Multilocus Sequence Typing (MLST) is based on the analysis of differences in housekeeping gene sequences to reveal relatively distant evolutionary processes to discriminate bacterial strains at the level of intraspecies or subspecies [111]. This method was first described by Maiden et al. (1998). Today, various MLST databases exist such as PubMLST (http://pubmlst.org/) or MLST (www.mlst.net/) [112,113].

Several housekeeping genes are used to study intraspecies relationships of LAB (*fus*A, *gdh*, *gyr*B, *ddl*, *mut*S, *pur*K1, *pgm*, *hsp*60, *ile*S, *pyr*G, *rec*A, *rec*G) [102,113,114]. These genes are essential and part of the core genome. Tanigawa et al. (2011) were able to sub-specify species such as *L. delbrueckii* subsp. *bulgaricus* using seven of these housekeeping genes (*fus*A, *gyr*B, *hsp*60, *ile*S, *pyr*G, *rec*A and *rec*G) [113]. However, Adimpong et al. (2013) demonstrated that MLST and splits-decomposition analyses of ribosomal RNA could not detect the correct subspecies level of the used *L. delbrueckii* strain ZN7a-9 T (type strain = DSM 26046 T = LMG 27067 T) [115].

Several publications are available providing information on different gene sequences for identification and classification of members of the genus *Bifidobacterium* (*tuf*, *rec*A, *xfp*, *atpD*, *groEL*, *groES*, *dnaK*, *hsp*60, *clpC*, *dnaB*, *dnaG*, *dnaJ1*, *purF*, *rpoC*) [116]. These target genes as well as *pyk* and *tal* have been studied and proven as useful for species and subspecies identification

of bifidobacteria [117]. This method was also successfully used to identify *L. casei* [118], *L. plantarum* [119] and *L. sanfranciscensis* [120]. In another publication 16 *L. plantarum* strains were identified by MLST, RFLP and 16S-23S rDNA analysis [119]. In this study MLST offered a much better discriminatory power than RFLP technique utilizing ITS regions showing 14 different allelic combinations within all 16 *L. plantarum* strains [119].

In comparison, the RAPD and the MLST method provide high resolutions [113]. Though, sub-specification of different LAB species in food is possible using MLST technique [113,121]. However, MLST is too laborious and time-consuming to use it for the analysis of a large number of strains in daily diagnostics.

Simple Sequence Repeats (SSR)
Loci with high mutation rates located in the genome of strains are useful for bacterial species typing using Simple Sequence Repeats (SSR) [111]. As an example, many SSR tracts are distributed and highly abundant in the genome of *L. johnsonii* NCC533 [111]. These loci are located both in the coding and non-coding regions and deliver the largest number of repeats for genetic characterization [111]. SSR regions of bacteria offer high discrimination power for phylogenetic analysis [111]. In combination with MLST the SSR technique is effective for typing, has high resolution and discriminative power differing between bacterial isolates from same animal species origin to level of subspecies [111].

Whole genome sequencing (WGS)
To get an inside view of genetic and structural variations of sequenced individuals for functional and comparative genomic studies whole genome sequencing or high throughput sequencing is the tool of choice [5,122]. PCR amplicons of the DNA of interest are fixed to beads which are sequenced using high throughput sequencing technologies. Next generation sequencing (NGS) enables sequencing processes in parallel producing thousands and millions of short sequence reads simultaneously [123]. Nowadays, high-throughput sequencing technologies are routinely used in biology and medicine to answer important genetically based questions [124]. New approaches were developed by advanced sequencing technologies such as the analysis of metabolic capacities, genome structure and variation analysis of different strains [124]. Being less expensive than formerly used genome sequencing techniques high-throughput sequencing technologies are widely used in industry. Recently, massively parallel sequencing or ultra-high-throughput sequencing (UHTS) offers thousands of sequencing-by-synthesis operations to be run at once [123]. Due to comparably low costs required for UHTS, it is widely used in commercial and academic approaches.

454 sequencing was the first high-throughput sequencing system to become available in the market based on pyrosequencing approach which was developed by Pål Nyrén and Mostafa Ronaghi in 1996 [125]. Expanding the possibilities of Sanger sequencing by using pyrosequencing read outs are simultaneously done by producing light whenever a nucleotide is incorporated [126]. High-throughput sequencing was possible after improving speed and power in computer technologies. The release of light and enhancement of technical analysis even facilitates sequencing in parallel [127]. Additionally, further read outs of the nucleotide structure using electrophoresis are not needed any longer [126].

WGS also offers an overview regarding evolutionary background and divergence of LAB strains belonging to one species [128]. It was shown that LAB genomes have reduced capacity encoding biosynthetic enzymes caused by adaptation to nutrient-rich environments [104]. Approximately, 600–1,200 genes were lost during evolution from their ancestor *Bacillus* including genes for sporulation [128]. Likewise, other genes involved in amino acid transport and peptidases have been duplicated to assure exploring protein-rich environments [128,129].

More and more whole genome sequencing is used to sequence genomes of reference strains using these data for a rapid and secure identification of strains in routine sequence analysis. In addition, sequence data of different mutants of a strain are screened easily assuring an optimal purpose. For instance, WGS revealed that *L. delbrueckii* subsp. *bulgaricus* and *Streptococcus* (*S.*) *thermophilus* should be used in combination as starting cultures to run fermentation processes in milk products [128]. By screening WGS data metabolic capabilities of both species revealed that they are dependent on each other to promote maximum growth potential due to the facility of *L. delbrueckii* subsp. *bulgaricus* to run the complete folate biosynthesis pathway [128]. In contrast, it lacks p-aminobenzoic acid (PABA) production offered by *S. thermophilus* [128,130].

WGS nowadays is commonly used in the food industry as a basis to identify regulatory mechanisms of secondary metabolite overproduction and subsequently improve fermentation processes of products [128]. More rapid fermentation processes reduce incubation time and manufacturers' costs in creating high quality products [128]. Cogan et al. (2007) demonstrated that genome sequencing offers a fast technique for the analysis of proteolytic abilities of *L. helveticus* CNRZ32 which plays an important role in cheese ripening [131]. Twelve genes were discovered which encode for specific proteolytic enzymes [131].

This method plays a significant role in screening metabolic properties of strains used in food fermentation processes. Thus, it is possible to arrange mutualistic or commensal relationships of starter cultures such as *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* [132,133].

In future, WGS will become more important in understanding the evolutionary, functional and physiological aspects of model organisms in medicine, pharmacy and natural sciences due to the fact that costs for WGS will decrease more and more. Thus, strain identification using WGS will increase in future. Even the metabolism and the function of non-cultivatable strains of the human or animal's microbiome will become decoded. Thus, knowledge about the interaction of microbiota enables abundant possibilities in reducing costs in treatment of e.g. disorders in the gut. To get an inside view of genetic and structural variations of sequenced individuals for functional and comparative genomic studies whole genome sequencing or high throughput sequencing is the tool of choice, although it is a time-consuming method (Table 1) [5,122].

Discussion & future trends

The aim of this review was to summarize methods and techniques used for the identification of probiotic lactobacilli. It is necessary to detect and to identify probiotics in food due to quality management reasons. Besides advertised strains other probiotic species are found in the same product as they are used as starter cultures running fermentation processes. Therefore, they should be mentioned in the description of the product due to their possible influences on the hosts' health [31,44,134]. It is necessary to screen products containing probiotics by official authorities or manufacturers thereby assuring the quality of the used probiotic strains (correct and viable strains, correct amount of cells, etc.). As an example, a recommended amount of viable probiotic bacterial cells has been defined between 10^6 to 10^8 cfu/ml by different agencies for humans consumption benefitting the immune response for the suppression of allergic and autoimmune disease [9,10]. Methods used for the analysis of probiotic bacteria in food have to be rapid, inexpensive and sensitive with the ability to quantify species of interest.

Some of the previously described methods can be utilized for the identification of lactobacilli at the species level (Table 1). Culture dependent methods (morphology, API 50 CHL, etc.) are well established, however, they are labor-intensive and often do not produce reliable results. Cultivation and isolation of strains from food is generally time-consuming and labor-intensive. The time needed for the designation of lactobacilli species varies between 48 h up to 96 h. It is impossible to identify colonies to the level of genus or species by morphology screening even if colonies are sub-cultivated for additional time periods. A genus, species or sub-species identification

is rarely possible. Therefore, culture-dependent isolation only offers a starting point for further investigations of the microbial composition of a product.

It is possible to analyze phenotypic abilities of lactobacilli after isolating them from food. Nevertheless, subspecies detection or quantification is not feasible using API 50 CHL stripes or BIOLOG system. In addition, both methods are time-consuming and labor-intensive and may also lead to misidentified or non-interpreted results in the case of the API 50 CHL system. Another technique for designation of bacteria is analyzing cell wall proteins by Fourier Transformation Infra-Red spectrometry (FTIR). This technology enables a closer insight at the species level instead of morphology screening (level of genus designation, Table 1). When sufficient protein structures will have been included in FTIR databases it is a potential tool to identify bacteria using proteins such as the S-layer of their cell surface. Reliable data of S-layer proteins are known for species used as probiotic additives. These data can be used for strain identification by FTIR which is an inexpensive, rapid and sensitive diagnostic approach. Different FTIR databases are available (http://www.fdmspectra.com/). Limitations of this method have been described when identifying members of the *L. casei* group, a fact which is caused by high genetic similarity of the species belonging to this group.

Another procedure is MALDI-TOF MS being increasingly used for the determination of bacterial species. Both methods – FTIR and MALDI-TOF MS – need approximately the same time for analysis (~ 49 h, Table 1). Furthermore, MALDI-TOF MS permits a rapid and sensitive identification to subspecies level, if protein data are available in a database. However, future research should lead to an increased specificity and sensitivity of both methodologies.

In contrast, culture-independent analysis by utilizing DNA directly isolated from the source of choice is less expensive, less time-consuming than MALDI-TOF MS and enables the user to identify and quantify bacteria down to level of strains (Table 1). A designation of strains within 36 h is possible using specific primers of 16S/23S-5S rRNA and by sequencing PCR products (Table 1). Species identification by sequencing is prolonged for additional 24 h increasing the amount of time needed (Table 1). Nearly the same time is necessary to subtype bacteria from food by MLST.

To our knowledge there is only one method available which offers – besides the identification approach – a second function: quantification of bacterial cells. The qPCR technique delivers all necessary requirements for being useful in daily diagnostic labs. It is rapid, inexpensive, culture-independent and easy to handle. It enables identification and quantification of bacterial cells within one workday (7 h, Table 1) using DNA mixtures directly extracted from food origin (Table 1). In contrast to methods previously described, qPCR does not require a second step of analysis to confirm the results, if primers are validated as specific to their target gene. Thus, qPCR has the potential being one of the most used methods to identify and quantify bacteria within a matrix of interest.

Comparing PCR-DGGE and 16S/23S-5S rRNA PCR plus additional sequencing, PCR-DGGE takes less time (Table 1). While enabling screening of a huge number of samples, it offers limited quantification power. This technique is predestined as being used in daily diagnostics, although, identification of some very closely related species such as *L. casei* / *L. paracasei* is not possible. The RAPD method offers a rapid identification of bacteria. However, Plengvidhya et al. (2004) made clear that it is not useful as a stand-alone method due to lack of reproducibility. Without sequencing, detection of probiotic lactobacilli from PCR amplicons is possible to the species level within 7 h using Single-strand conformation polymorphisms (SSCP). The method itself is accurate and sensitive, even without additional sequencing, although other methods are advised to confirm the results.

A possibility to identify members of the genus *Lactobacillus* may be combining SSR analysis with MLST. However, combining two different methods increases time, labor-intensity and costs. Currently, only few publications are available detecting LAB using SSR technology.

WGS technique itself represents a method which is more and more available on the market due to decreasing costs and their wide range of possibilities by generating whole genome sequencing data. By having these data other techniques become affordable such as designing species-specific primers for qPCR or other molecular based techniques to identify and quantify bacterial strains. In addition, the metabolic potential and abilities of a given strain is available for industrial usage and a much more rapid screening for physiological, evolutionary and functional capabilities is possible. Thus, WGS will become more important for strain identification in future, if costs will decrease steadily. However, it is a time-consuming technique (40–88 h, Table 1), however offers an inside view into the strain's physiological properties.

In the future, if it is not possible to establish any other identification tool or techniques to analyze probiotics in a much faster way, WGS and real time PCR will become important as rapid tools identifying, screening and analyzing probiotic bacteria compositions. In addition, the availability of both methods supported by decreasing costs will increase their usage within the coming years.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions

SRH designed, structured and prepared the manuscript. In addition, he discussed and interpreted the results. He was also the one who 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.

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References

1. Reiss T: **Fictions of the cosmos: science and literature in the seventeenth century.** *Isis* 2013, **104**(1):158–159.
2. Zhang ZG, et al: **Phylogenomic reconstruction of lactic acid bacteria: an update.** *BMC Evol Biol* 2011, **11**:1.
3. Tannock GW: **A special fondness for lactobacilli.** *Appl Environ Microbiol* 2004, **70**(6):3189–3194.
4. Coeuret V, et al: **Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products.** *Lait* 2003, **83**(4):269–306.
5. Grover S, et al: **Probiotics for human health - new innovations and emerging trends.** *Gut Pathogens* 2012, **4**(15):1–4.
6. Tamani RJ, Goh KKT, Brennan CS: **Physico-chemical properties of sourdough bread production using selected lactobacilli starter cultures.** *J Food Qual* 2013, **36**(4):245–252.
7. Pal K, et al: **Comparison and evaluation of molecular methods used for identification and discrimination of lactic acid bacteria.** *J Sci Food Agric* 2012, **92**(9):1931–1936.
8. Lalitha TA: **Probiotics and oral health.** *J Oral Res Rev* 2011, **3**(1):20–26.
9. Sarkar S: **Microbiological considerations for probiotic supplemented foods.** *Int J Microbiol Adv Immunol* 2013, **1**(1):1–7.
10. Shah NP: **Probiotic bacteria: selective enumeration and survival in dairy foods.** *J Dairy Sci* 2000, **83**(4):894–907.
11. EC: **Corrigendum to Regulation (EC) No 1924/2006 of the European Parliament and of the Council of 20 December 2006 on nutrition and health claims made on foods (Official Journal of the European Union L 404 of 30 December 2006).** *Off J Eur Union* 2007, **L**12:3–18.
12. Margolles A, Mayo B, Ruas-Madiedo P: **Screening, identification, and characterization of lactobacillus and bifidobacterium strains.** In *Handbook of probiotics and prebiotics.* Edited by Nomoto K, Salminen S, Lee YK. Hoboken, New Jersey: Wiley; 2009:15.
13. Ivanova P, et al: **Molecular typing by genus-specific PCR and RAPD profiling of diverse Lactobacillus delbrueckii strains isolated from cow, sheep and buffalo yoghurts.** *Biotechnol Biotechnol Equip* 2008, **22**(2):748–753.
14. Danner H, et al: **Acetic acid increases stability of silage under aerobic conditions.** *Appl Environ Microbiol* 2003, **69**(1):562–567.
15. Song YL, et al: **Rapid identification of 11 human intestinal Lactobacillus species by multiplex PCR assays using group- and species-specific primers derived from the 16S-23S rRNA intergenic spacer region and its flanking 23S rRNA.** *FEMS Microbiol Lett* 2000, **187**(2):167–173.
16. Fuller R: **Probiotics in man and animals.** *J Appl Bacteriol* 1989, **66**(05):365–378.
17. Schrezenmeir J, de Vrese M: **Probiotics, prebiotics, and synbiotics - approaching a definition.** *Am J Clin Nutr* 2001, **73**(2):361s–364s.
18. Reid G, Bocking A: **The potential for probiotics to prevent bacterial vaginosis and preterm labor.** *Am J Obstet Gynecol* 2003, **189**(4):1202–1208.
19. Calabrese V, et al: **In vivo induction of heat shock proteins in the substantia nigra following L-DOPA administration is associated with increased activity of mitochondrial complex I and nitrosative stress in rats: regulation by glutathione redox state.** *J Neurochem* 2007, **101**(3):709–717.
20. Frizzo LS, et al: **The effect of supplementation with three lactic acid bacteria from bovine origin on growth performance and health status of young calves.** *J Anim Vet Adv* 2008, **7**(4):400–408.
21. Nagpal R, et al: **Probiotics, their health benefits and applications for developing healthier foods: a review.** *Fems Microbiol Lett* 2012, **334**(1):1–15.
22. Mugambi MN, et al: **Probiotics, prebiotics infant formula use in preterm or low birth weight infants: a systematic review.** *Nutr J* 2012, **11**(58):1–18.
23. Makinen K, et al: **Science and technology for the mastership of probiotic applications in food products.** *J Biotechnol* 2012, **162**(4):356–365.
24. Jiménez G, et al: **Description of Bacillus toyonensis sp. nov., a novel species of the Bacillus cereus group, and pairwise genome comparisons of the species of the group by means of ANI calculations.** *Syst Appl Microbiol* 2013, **36**(6):383–391.
25. Salvetti E, et al: **Evolution of lactic acid bacteria in the order Lactobacillales as depicted by analysis of glycolysis and pentose phosphate pathways.** *Syst Appl Microbiol* 2013, **36**:291–305.
26. Singh Y, et al: **Emerging importance of holobionts in evolution and in probiotics.** *Gut Pathogens* 2013, **5**(12):1–8.
27. Burgain J, et al: **Encapsulation of probiotic living cells: from laboratory scale to industrial applications.** *J Food Eng* 2011, **104**(4):467–483.
28. Jagerbrink T, et al: **Differential protein expression in pancreatic islets after treatment with an imidazoline compound.** *Cell Mol Life Sci* 2007, **64**(10):1310–1316.
29. Dubois D, et al: **Performances of the Vitek MS matrix-assisted laser desorption ionization-time of flight mass spectrometry system for rapid identification of bacteria in routine clinical microbiology.** *J Clin Microbiol* 2012, **50**(8):2568–2576.
30. Larue RW, et al: **Chlamydial Hsp60-2 is iron responsive in Chlamydia trachomatis serovar E-infected human endometrial epithelial cells in vitro.** *Infect Immun* 2007, **75**(5):2374–2380.
31. Saad N, et al: **An overview of the last advances in probiotic and prebiotic field.** *Lwt-Food Sci Technol* 2013, **50**(1):1–16.
32. Gao XW, et al: **Dose–response efficacy of a proprietary probiotic formula of lactobacillus acidophilus CL1285 and *Lactobacillus casei* LBC80R for antibiotic-associated diarrhea and clostridium difficile-associated diarrhea prophylaxis in adult patients.** *Am J Gastroenterol* 2010, **105**(7):1636–1641.
33. Divya JB, et al: **Probiotic fermented foods for health benefits.** *Eng Life Sci* 2012, **12**(4):377–390.
34. Bested AC, Logan AC, Selhub EM: **Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: Part II - contemporary contextual research.** *Gut Pathog* 2013, **5**(3):1–14.
35. Thomas LV, Ockhuizen T: **New insights into the impact of the intestinal microbiota on health and disease: a symposium report.** *Br J Nutr* 2012, **107**:S1–S13.
36. Mobili P, et al: **Characterization of S-layer proteins of Lactobacillus by FTIR spectroscopy and differential scanning calorimetry.** *Vib Spectrosc* 2009, **50**(1):68–77.
37. Wiegel J, Tanner R, Rainey FA: **An introduction to the family clostridiaceae.** In *The prokaryotes: Vol. 4: bacteria: firmicutes, cyanobacteria.* 4th edition. Edited by Martin Dworkin M, et al. Springer-Verlag Berlin Heidelberg: Springer Science + Business Media, LLC; 2006:666.
38. Ciorba MA: **A Gastroenterologist's guide to probiotics.** *Clin Gastroenterol Hepatol* 2012, **10**(9):960–968.
39. Bille E, et al: **MALDI-TOF MS Andromas strategy for the routine identification of bacteria, mycobacteria, yeasts Aspergillus spp. and positive blood cultures.** *Clin Microbiol Infect* 2012, **18**(11):1117–1125.
40. Klaenhammer TR, et al: **The impact of probiotics and prebiotics on the immune system.** *Nat Rev Immunol* 2012, **12**(10):728–734.
41. Black BA, et al: **Antifungal lipids produced by lactobacilli and their structural identification by normal phase LC/Atmospheric pressure photoionization-MS/MS.** *J Agric Food Chem* 2013, **61**(22):5338–5346.
42. Jacobi CA, Schulz C, Malfertheiner P: **Treating critically ill patients with probiotics: Beneficial or dangerous?** *Gut Pathog* 2011, **3**(2):1–5.
43. Ingrassia I, Leplingard A, Darfeuille-Michaud A: **Lactobacillus casei DN-114 001 inhibits the ability of adherent-invasive Escherichia coli isolated from Crohn's disease patients to adhere to and to invade intestinal epithelial cells.** *Appl Environ Microbiol* 2005, **71**(6):2880–2887.

44. Rajasekaran A, Kalaivani M: **Designer foods and their benefits: a review.** *J Food Sci Technol-Mysore* 2013, **50**(1):1–16.

45. van Belkum A, *et al*: **Biomedical mass spectrometry in today's and tomorrow's clinical microbiology laboratories.** *J Clin Microbiol* 2012, **50**(5):1513–1517.

46. Alvarez-Olmos MI, *et al*: **Vaginal lactobacilli in adolescents - presence and relationship to local and systemic immunity, and to bacterial vaginosis.** *Sex Transm Dis* 2004, **31**(7):393–400.

47. Reeves E: **Fictions of the cosmos: science and literature in the seventeenth century.** *Stud Hist Philos Sci* 2012, **43**(3):421–424.

48. Brolazo EM, *et al*: **Correlation between Api 50 Ch and multiplex polymerase chain reaction for the identification of vaginal lactobacilli in isolates.** *Braz J Microbiol* 2011, **42**(1):225–232.

49. Boyd MA, Antonio MA, Hillier SL: **Comparison of API 50 CH strips to whole-chromosomal DNA probes for identification of Lactobacillus species.** *J Clin Microbiol* 2005, **43**(10):5309–5311.

50. Nagy E, Petterson M, Mardh PA: **Antibiosis between bacteria isolated from the vagina of women with and without signs of bacterial vaginosis.** *Apmis* 1991, **99**(8):739–744.

51. Nigatu A: **Evaluation of numerical analyses of RAPD and API 50 CH patterns to differentiate Lactobacillus plantarum, Lact. fermentum, Lact. rhamnosus, Lact. sake, Lact. parabuchneri, Lact. gallinarum, Lact. casei, Weissella minor and related taxa isolated from kocho and tef.** *J Appl Microbiol* 2000, **89**(6):969–978.

52. Tynkkynen S, *et al*: **Comparison of ribotyping, randomly amplified polymorphic DNA analysis, and pulsed-field gel electrophoresis in typing of Lactobacillus rhamnosus and L-casei strains.** *Appl Environ Microbiol* 1999, **65**(9):3908–3914.

53. Biolog Inc.: *Biolog, Anaerobe Identification Test Panel*. Calofornia, USA: AN MicroPlate™; 2007.

54. Williams AG, Withers SE, Banks JM: **Energy sources of non-starter lactic acid bacteria isolated from Cheddar cheese.** *Int Dairy J* 2000, **10**(1–2):17–23.

55. Di Cagno R, *et al*: **Genotypic and phenotypic diversity of Lactobacillus rossiae strains isolated from sourdough.** *J Appl Microbiol* 2007, **103**(4):821–835.

56. De Angelis M, *et al*: **Molecular and functional characterization of Lactobacillus sanfranciscensis strains isolated from sourdoughs.** *Int J Food Microbiol* 2007, **114**(1):69–82.

57. Di Cagno R, *et al*: **Effect of autochthonous lactic acid bacteria starters on health-promoting and sensory properties of tomato juices.** *Int J Food Microbiol* 2009, **128**(3):473–483.

58. Tamang B, *et al*: **Phenotypic and genotypic identification of lactic acid bacteria isolated from ethnic fermented bamboo tender shoots of North East India.** *Int J Food Microbiol* 2008, **121**(1):35–40.

59. Dziuba B, *et al*: **Identification of lactic acid bacteria using FTIR spectroscopy and cluster analysis.** *Int Dairy J* 2007, **17**(3):183–189.

60. Dziuba B, Nalepa B: **Identification of lactic acid bacteria and propionic acid bacteria using FTIR spectroscopy and artificial neural networks.** *Food Technol Biotechnol* 2012, **50**(4):399–405.

61. Spagnoli LG, *et al*: **Persistent Chlamydia pneumoniae infection of cardiomyocytes is correlated with fatal myocardial infarction.** *Am J Pathol* 2007, **170**(1):33–42.

62. Bauer R, *et al*: **FTIR spectroscopy for grape and wine analysis.** *Anal Chem* 2008, **80**(5):1371–1379.

63. Maquelin K, *et al*: **Prospective study of the performance of vibrational spectroscopies for rapid identification of bacterial and fungal pathogens recovered from blood cultures.** *J Clin Microbiol* 2003, **41**(1):324–329.

64. Prabhakar V, *et al*: **Classification of Swiss cheese starter and adjunct cultures using Fourier transform infrared microspectroscopy.** *J Dairy Sci* 2011, **94**(9):4374–4382.

65. Samelis J, *et al*: **FTIR-based polyphasic identification of lactic acid bacteria isolated from traditional Greek Graviera cheese.** *Food Microbiol* 2011, **28**(1):76–83.

66. Duskova M, *et al*: **Identification of lactobacilli isolated from food by genotypic methods and MALDI-TOF MS.** *Int J Food Microbiol* 2012, **159**(2):107–114.

67. Lay JO: **MALDI-TOF mass spectrometry of bacteria.** *Mass Spectrom Rev* 2001, **20**(4):172–194.

68. Farfour E, *et al*: **Evaluation of the andromas matrix-assisted laser desorption ionization-time of flight mass spectrometry system for identification of aerobically growing gram-positive bacilli.** *J Clin Microbiol* 2012, **50**(8):2702–2707.

69. O'Sullivan DJ: **Techniques for microbial species identification and characterization to identify commercially important traits.** *Improving the Flavour of Cheese* 2007, **142**:199–218.

70. Jiang HF, *et al*: **Heat shock protein 70 is translocated to lipid droplets in rat adipocytes upon heat stimulation.** *Biochimica Et Biophysica Acta-Mol Cell Biol Lipids* 2007, **1771**(1):66–74.

71. Sato H, *et al*: **Characterization of the Lactobacillus casei group based on the profiling of ribosomal proteins coded in S10-spc-alpha operons as observed by MALDI-TOF MS.** *Syst Appl Microbiol* 2012, **35**(7):447–454.

72. Angelakis E, *et al*: **Rapid and accurate bacterial identification in probiotics and yoghurts by MALDI-TOF mass spectrometry.** *J Food Sci* 2011, **76**(8):M568–M572.

73. Carbonnelle E, *et al*: **MALDI-TOF mass spectrometry tools for bacterial identification in clinical microbiology laboratory.** *Clin Biochem* 2011, **44**(1):104–109.

74. Bested AC, Logan AC, Selhub EM: **Intestinal microbiota, probiotics and mental health: from Metchnikoff to modern advances: Part I - autointoxication revisited.** *Gut Pathog* 2013, **5**:1–16.

75. Danielson D: **Fictions of the cosmos: science and literature in the seventeenth century.** *J Hist Astron* 2012, **43**:364–366.

76. Luo Y, *et al*: **Identification and characterization of lactic acid bacteria from forest musk deer feces.** *Afr J Microbiol Res* 2012, **6**(29):5871–5881.

77. Ouoba LII, *et al*: **Genotypic diversity of lactic acid bacteria isolated from African traditional alkaline-fermented foods.** *J Appl Microbiol* 2010, **108**(6):2019–2029.

78. Fleck ZC, *et al*: **Identification of lactic acid bacteria isolated from dry fermented sausages.** *Veterinarski Arhiv* 2012, **82**(3):265–272.

79. Fujimoto J, Watanabe K: **Quantitative detection of viable bifidobacterium bifidum BF-1 cells in human feces by using propidium monoazide and strain-specific primers.** *Appl Environ Microbiol* 2013, **79**(7):2182–2188.

80. Castoldi M, *et al*: **Expression profiling of MicroRNAs by quantitative real-time PCR.** In *PCR technology: current innovations.* Edited by Nolan T, Bustin SA. Boca Raton: CRC Press, Taylor & Francis Group; 2013.

81. Bustin SA, Zaccara S, Nolan T: **An introduction to the real-time polymerase chain reaction**. In *Quantitative real-time PCR in applied microbiology.* Edited by Filion M. Norfolk, UK: Caister Academic Press; 2012.

82. Meng HM, *et al*: **Efficient fluorescence turn-on probe for zirconium via a target-triggered DNA molecular beacon strategy.** *Anal Chem* 2012, **84**(5):2124–2128.

83. Miller DM, Dudley EG, Roberts RF: **Technical note: development of a quantitative PCR method for monitoring strain dynamics during yogurt manufacture.** *J Dairy Sci* 2012, **95**(9):4868–4872.

84. Sohier D, *et al*: **Polyphasic approach for quantitative analysis of obligately heterofermentative Lactobacillus species in cheese.** *Food Microbiol* 2012, **31**(2):271–277.

85. Postollec F, *et al*: **Recent advances in quantitative PCR (qPCR) applications in food microbiology.** *Food Microbiol* 2011, **28**(5):848–861.

86. Junick J, Blaut M: **Quantification of human fecal bifidobacterium species by use of quantitative real-time PCR analysis targeting the groEL gene.** *Appl Environ Microbiol* 2012, **78**(8):2613–2622.

87. Logan AC, Rao AV, Irani D: **Chronic fatigue syndrome: lactic acid bacteria may be of therapeutic value.** *Med Hypotheses* 2003, **60**(6):915–923.

88. Chen TT, *et al*: **Identification of bacterial strains in viili by molecular taxonomy and their synergistic effects on milk curd and exopolysaccharides production.** *Afr J Biotechnol* 2011, **10**(74):16969–16975.

89. Florez AB, Mayo B: **Microbial diversity and succession during the manufacture and ripening of traditional, Spanish, blue-veined Cabrales cheese, as determined by PCR-DGGE.** *Int J Food Microbiol* 2006, **110**(2):165–171.

90. Cocolin L, *et al*: **Denaturing gradient gel electrophoresis analysis of the 16S rRNA gene V1 region to monitor dynamic changes in the bacterial population during fermentation of Italian sausages.** *Appl Environ Microbiol* 2001, **67**(11):5113–5121.

91. Renouf V, *et al*: **Lactic acid bacteria evolution during winemaking: Use of rpoB gene as a target for PCR-DGGE analysis.** *Food Microbiol* 2006, **23**(2):136–145.

92. Randazzo CL, *et al*: **Bacterial population in traditional sourdough evaluated by molecular methods.** *J Appl Microbiol* 2005, **99**(2):251–258.

93. van Beek S, Priest FG: **Evolution of the lactic acid bacterial community during malt whisky fermentation: A polyphasic study.** *Appl Environ Microbiol* 2002, **68**(1):297–305.

94. Liu WJ, *et al*: **Isolation and identification of lactic acid bacteria from Tarag in Eastern Inner Mongolia of China by 16S rRNA sequences and DGGE analysis.** *Microbiol Res* 2012, **167**(2):110–115.
95. Fontana C, *et al*: **Surface microbiota analysis of Taleggio, Gorgonzola, Casera, Scimudin and Formaggio di Fossa Italian cheeses.** *Int J Food Microbiol* 2010, **138**(3):205–211.
96. Gonzalez JM, *et al*: **An efficient strategy for screening large cloned libraries of amplified 16S rDNA sequences from complex environmental communities.** *J Microbiol Methods* 2003, **55**(2):459–463.
97. Fujimoto J, *et al*: **Identification and quantification of Lactobacillus casei strain Shirota in human feces with strain-specific primers derived from randomly amplified polymorphic DNA.** *Int J Food Microbiol* 2008, **126**(1–2):210–215.
98. Logan AC, Katzman M: **Major depressive disorder: probiotics may be an adjuvant therapy.** *Med Hypotheses* 2005, **64**(3):533–538.
99. Satokari RM, *et al*: **Molecular approaches for the detection and identification of bifidobacteria and lactobacilli in the human gastrointestinal tract.** *Syst Appl Microbiol* 2003, **26**(4):572–584.
100. Cocconcelli PS, *et al*: **Use of RAPD and 16S rDNA sequencing for the study of Lactobacillus population dynamics in natural whey culture.** *Lett Appl Microbiol* 1997, **25**(1):8–12.
101. Cremonesi P, *et al*: **Development of a pentaplex PCR assay for the simultaneous detection of Streptococcus thermophilus, Lactobacillus delbrueckii subsp bulgaricus, L. delbrueckii subsp. lactis, L. helveticus, L. fermentum in whey starter for Grana Padano cheese.** *Int J Food Microbiol* 2011, **146**(2):207–211.
102. Gosiewski T, *et al*: **The application of genetics methods to differentiation of three Lactobacillus species of human origin.** *Ann Microbiol* 2012, **62**(4):1437–1445.
103. Plengvidhya V, Breidt F, Fleming HP: **Use of RAPD-PCR as a method to follow the progress of starter cultures in sauerkraut fermentation.** *Int J Food Microbiol* 2004, **93**(3):287–296.
104. Kneifel W, Domig KJ: **Taxonomie von Milchsäurebakterien mit probiotischer Kapazität.** In *Probiotika, präbiotika und synbiotika.* Edited by Bischoff SC. Stuttgart: Georg Thieme Verlag KG; 2009:103–117.
105. Pingault NM, *et al*: **A comparison of molecular typing methods for Moraxella catarrhalis.** *J Appl Microbiol* 2007, **103**(6):2489–2495.
106. Naimuddin M, Nishigaki K: **Genome analysis technologies: towards species identification by genotype.** *Brief Func Genomic Proteomics* 2003, **1**(4):356–371.
107. Smalla K, *et al*: **Bacterial diversity of soils assessed by DGGE, T-RFLP and SSCP fingerprints of PCR-amplified 16S rRNA gene fragments: Do the different methods provide similar results?** *J Microbiol Meth* 2007, **69**(3):470–479.
108. Callon C, *et al*: **Stability of microbial communities in goat milk during a lactation year: molecular approaches.** *Syst Appl Microbiol* 2007, **30**(7):547–560.
109. Martin-Platero AM, *et al*: **Polyphasic study of microbial communities of two Spanish farmhouse goats' milk cheeses from Sierra de Aracena.** *Food Microbiol* 2009, **26**(3):294–304.
110. An C, *et al*: **Comparison of PCR-DGGE and PCR-SSCP analysis for bacterial flora of Japanese traditional fermented fish products, aji-narezushi and iwashi-nukazuke.** *J Sci Food Agric* 2010, **90**(11):1796–1801.
111. Buhnik-Rosenblau K, *et al*: **Indication for Co-evolution of Lactobacillus johnsonii with its hosts.** *Bmc Microbiol* 2012, **12**(149):1–10.
112. Maiden MCJ, *et al*: **Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms.** *Proc Natl Acad Sci U S A* 1998, **95**(6):3140–3145.
113. Tanigawa K, Watanabe K: **Multilocus sequence typing reveals a novel subspeciation of Lactobacillus delbrueckii.** *Microbiology* 2011, **157**(Pt 3):727–738.
114. Strus M, *et al*: **Studies on the effects of probiotic Lactobacillus mixture given orally on vaginal and rectal colonization and on parameters of vaginal health in women with intermediate vaginal flora.** *Eur J Obstet Gynecol Reprod Biol* 2012, **163**(2):210–215.
115. Adimpong DB, *et al*: **Lactobacillus delbrueckii subsp. jakobsenii subsp. nov., isolated from dolo wort; an alcoholic fermented beverage in Burkina Faso.** *Int J Syst Evol Microbiol* 2013. doi:10.1099/ijs.0.048769-0 (accepted version).
116. Ventura M, *et al*: **Analysis of bifidobacterial evolution using a multilocus approach.** *Int J Syst Evol Microbiol* 2006, **56**:2783–2792.
117. Vaugien L, Prevots F, Roques C: **Bifidobacteria identification based on 16S rRNA and pyruvate kinase partial gene sequence analysis.** *Anaerobe* 2002, **8**(6):341–344.
118. Cai H, *et al*: **Genotypic and phenotypic characterization of Lactobacillus casei strains isolated from different ecological niches suggests frequent recombination and niche specificity.** *Microbiol-Sgm* 2007, **153**:2655–2665.
119. de las Rivas B, Marcobal A, Munoz R: **Development of a multilocus sequence typing method for analysis of Lactobacillus plantarum strains.** *Microbiol-Sgm* 2006, **152**:85–93.
120. Picozzi C, *et al*: **Genetic diversity in Italian Lactobacillus sanfranciscensis strains assessed by multilocus sequence typing and pulsed-field gel electrophoresis analyses.** *Microbiol-Sgm* 2010, **156**:2035–2045.
121. Tanganurat W, *et al*: **Genotypic and phenotypic characterization of Lactobacillus plantarum strains isolated from Thai fermented fruits and vegetables.** *J Basic Microbiol* 2009, **49**(4):377–385.
122. Durbin RM, *et al*: **A map of human genome variation from population scale sequencing.** *Nature* 2010, **467**:1061–1073.
123. Ansorge WJ: **Next-generation DNA sequencing techniques.** *N Biotechnol* 2009, **25**(4):195–203.
124. Soon WW, Hariharan M, Snyder MP: **High-throughput sequencing for biology and medicine.** *Mol Syst Biol* 2013, **9**(640):1–14.
125. Ronaghi M, *et al*: **Real-time DNA sequencing using detection of pyrophosphate release.** *Anal Biochem* 1996, **242**(1):84–89.
126. Kircher M, Kelso J: **High-throughput DNA sequencing - concepts and limitations.** *Bioessays* 2010, **32**(6):524–536.
127. Rothberg JM, Leamon JH: **The development and impact of 454 sequencing.** *Nat Biotechnol* 2008, **26**(10):1117–1124.
128. Pfeiler EA, Klaenhammer TR: **The genomics of lactic acid bacteria.** *Trends Microbiol* 2007, **15**(12):546–553.
129. Makarova KS, Koonin EV: **Evolutionary genomics of lactic acid bacteria.** *J Bacteriol* 2007, **189**(4):1199–1208.
130. van de Guchte M, *et al*: **The complete genome sequence of Lactobacillus bulgaricus reveals extensive and ongoing reductive evolution.** *Proc Natl Acad Sci U S A* 2006, **103**(24):9274–9279.
131. Cogan TM, *et al*: **Advances in starter cultures and cultured foods.** *J Dairy Sci* 2007, **90**(9):4005–4021.
132. Herve-Jimenez L, *et al*: **Postgenomic analysis of streptococcus thermophilus cocultivated in milk with lactobacillus delbrueckii subsp bulgaricus: involvement of nitrogen, purine, and iron metabolism.** *Appl Environ Microbiol* 2009, **75**(7):2062–2073.
133. Claesson MJ, van Sinderen D, O'Toole PW: **The genus Lactobacillus - a genomic basis for understanding its diversity.** *Fems Microbiol Lett* 2007, **269**(1):22–28.
134. Liong M: **Preface.** In *Probiotics: Biology, Genetics and Health Aspects – Microbiology Monographs.* Edited by Steinbüchel A. ; 2011.