8 SUMMARY

Development and application of 16S rRNA-targeted gene probes for the identification of biotechnologically used *Lactobacillus*-strains of the *L. acidophilus*- and *L. casei*-group

Due to numerous desirable metabolic properties lactobacilli have long been used as starter cultures in food production, as feed additives in animal nutrition, and as therapeutic agents in the pharmaceutical industry. Members of the *L. acidophilus*- and the *L. casei*-group are increasingly applicated as probiotics in food, thus requiring reliable and fast identification. The classical microbiological methods for identification of microorganisms to the species-level are usually tedious and sometimes provide ambiguous results due to varying strain forms within a species. In particular, the differentiation within the *L.* (= *Lactobacillu*s) *acidophilus*-group, e.g. between *L. crispatus* and *L. gasseri* resp. between *L. johnsonii* and *L. acidophilus* may be difficult. In the *L. casei*-group modifications in the taxonomic classification of species have caused considerable confusion.

Therefore, the intention of this study was the development of gene probes for the species *L. acidophilus, L. gasseri, and L. johnsonii (L. acidophilus*-group), and for *L. zeae*, *L. paracasei*, and *L. rhamnosus* (*L. casei*-group), which are commonly used as probiotics. Furthermore, the significance of gene probes was to be evaluated in comparison to other molecular methods.

For this purpose the databases and computer programs of the *NCBI/GenBank* (**N**ational **C**enter for **B**iotechnology **I**nformation) as well as the *RDP* (**R**ibosomal **D**atabase **P**roject) of the Michigan State University, USA, which can both be accessed via internet, were utilized. The synthesis of the *L. gasseri*-probe was performed with the PCR-technique after partial sequence analysis of the 16S rRNA-gene of a *L. gasseri*-reference strain (ATCC 19992 / DSM 20077). For the validation of the probes a total of 85 type- and reference strains out of the *L. acidophilus*- and the *L. casei*-group (type- / reference strains, isolates from dairy products, pharmaceutical preparations, and clinical material) as well as comparatively seven strains from other *Lactobacillus*-species and two *Weissella*-species were included. With regard to their classification, all tested strains were previously subjected to classical phenotypical testing. The molecular-based testing was performed by dot blot-hybridization.

The hybridization results demonstrated that gene probes can be applied for species-specific identification of biotechnologically used lactobacilli of these two groups. All tested strains belonging to the species *L. acidophilus* sensu stricto were exclusively detected with the *L. acidophilus*-probe. Cross-reactions occurred with the phylogenetically closely related species *L. gasseri* and *L. johnsonii*, i.e. some strains reacted additionally with another probe, which was not intended for the identification of the respective species. Thus, the *L. gasseri*-probe also detected the *L. johnsonii*-strains. Differentiation between these two species was possible by addtionally applying the *L. johnsonii*-probe, which only hybridized with the *L. johnsonii*-strains.

The type strain of *L. casei* (ATCC 393^T) reacted with the *L. paracasei*-probe as well as with the *L. zeae*-specific probe. Three additional strains, which were declared as members of *L. casei*, hybridized solely with the *L. paracasei*-probe as well as three *L. paracasei*-reference strains and one additional collection strain, which was declared as belonging to *L. paracasei*. This induced a (sub-) division of the *L. casei*-strains into two subgroups (*L. casei* I resp. II). These findings support a present proposal for the solution of the taxonomic problems within the *L. casei*-group, e.g. that the current *L. casei*-type strain is to be assigned to *L. zeae*, whereas other *L. casei*-strains (including strain ATCC 334 suggested as the neo-type strain of *L. casei* by several authors) may be grouped with *L. paracasei*.

Two gradations with respect to the hybridization reactions also appeared among strains phenotypically identified as *L. rhamnosus*. Approximately half of these strains (12) reacted only with the *L. rhamnosus*-probe, the other half (10) however reacted simultaneously with the *L. paracasei*-probe. All but one of the latter strains where of clinical origin. Due to these cross-reactions the assignment into the subgroups *L. rhamnosus* I resp. II was carried out. The *L. rhamnosus*-probe can nevertheless be regarded as species-specific. A special case was represented by a clinical strain phenotypically identified as *L. rhamnosus*, which reacted solely with the *L. paracasei*-probe. In other investigations, this strain was also identified as *L. casei* / *paracasei* by means of RAPD-PCR.

Cross-reactions with other species out of the *L. acidophilus*-group (*L. crispatus*, *L. amylovorus*, *L. gallinarum*) as well as with other representatives of lactic acid bacteria (*Lactobacillus* and *Weissella*) did not occur.

The comparison of the hybridization results with those obtained in previously performed investigations with other molecular methods with the same strains, confirms the significance of gene probes for the classification of strains.

The delimitation of *L. gasseri* from *L. crispatus* proves difficult with phenotypic methods. Both species belong to the autochthonous microflora and have special relevance in the context of their use as probiotics. Due to this fact, the application of additional gene probes is required. Although the synthesis and verification of a *L. crispatus*-probe was not feasible in the scope of these investigations, a comparison of *L. gasseri* and *L. crispatus* type strain-sequences retrieved by a BLAST-analysis (NCBI/GenBank) displayed two nucleotide segments, which could achieve a differentiation with the methodology used in this study.

Regarding the required work load, the hybridization-technique indicated advantages in comparison to other molecular-based methods, e.g. RAPD-PCR-technique. An unfavourable aspect of the technique applied hitherto was the fact that the dot blot procedure could be used only after having cultured the strains. Faster identification can be expected by the reverse dot blot-hybridization technique, where cultivation of strains prior to hybridization is not necessary and amplification of the target genes with specific primers requires hybridization periods of only 2-4 hours.

The hybridization technique is thus suitable for the reliable identification of some biotechnologically relevant *Lactobacillus*-species. Further probes, in particular for the species *L. crispatus* and *L. casei/paracasei* must be developed and validated. The applicability of these methods in the routine is still pending. However, on the basis of the results with collection strains originating from commercial products, the suitability for routine application can be expected.