USE OF REAL-TIME PCR METHOD FOR IDENTIFICATION OF MICROORGANISMS USED IN MEAT INDUSTRY

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Abstract – This paper describes the development of the method for the identification of lactic acid bacteria used in meat industry, namely Lactobacillus spp., using the real-time polymerase chain reaction (PCR). The primers for identification of species L. rhamnosus and L. casei were selected on the basis of constitutive genes; the PCR set-up modes were worked out; the expediency of their use was shown.

Key Words – real-time PCR, technological cultures, Lactobacillus spp.

- INTRODUCTION

Nowadays, meat industry widely uses the bacterial preparations, which composition could include one, two or more microbial species.

Starter cultures used in meat industry present liquid or lyophilized forms of microorganisms from different genera including Lactobacillus spp., Pediococcus spp., Staphylococcus spp. and Micrococcus spp. Lactic acid bacteria (LAB) could be starter, bioprotective or probiotic. With that, the manufacturer of the bacterial preparations specify their presence, as a rule, in a quantity less that the total quantity of LAB in a preparation, which currently makes it practically impossible to verify their presence in the bacterial preparation.
For complex starter cultures functioning, not only the total quantity of microorganisms is important but also the presence of all claimed microbial strains as well as the specified ratio between them in a starter culture since the contribution of each culture to the formation of the product properties can differ significantly. At present, however, the assessment of starter cultures in the Russian Federation is carried out by the determination of the quantity of the microorganisms of the declared microflora (not less than 10^9 CFU/cm^3 for cultures and 10^{10} CFU/cm^3 for concentrates).
Analysis of the literature data shows that at present primers are selected on the basis of 16S rRNA gene. We considered the possibility to use for the selection of species-specific genome sites, the so called housekeeping genes – the genes which are necessary for the maintenance of the basic life functions. They possess high resolving power to differentiate species and, as a rule, are single-copy, which afterwards would be necessary for the development of the quantification of the target microorganisms by real-time PCR.

- MATERIALS AND METHODS

The species-specific gene and its site were selected using the International genome database NCBI, the primer was designed on the basis of the selected DNA fragment with the use of the Primer Express software, the reaction was optimized on the basis of the concentration gradient of the melting temperature. DNA was extracted using the kit DNA-sorb-AM (InterLabService Company). Real-time PCR was performed on the AbiPrism analyzer with the use of the SYBR Green fluorescent dye.

- RESULTS AND DISCUSSION

The analysis of the literature data shows that at present primers are selected on the basis of 16S rRNA gene. We compared the degree of 16S rRNA gene similarity of these two microbial species by the on-line BLAST program which showed that, in general, the similarity of 16S rRNA gene of L. casei and L. rhamnosus was 99%. It suggests that it is not expedient to select species-specific primers on the basis of this DNA site because of the high possibility of false positive cross reactions with closely related species of LAB.

The comparative assessment of the genetic distances of the target sites of 16S rRNA gene and gyrB gene (Fig. 1a, b) of several LAB species was carried out.

It can be seen from the figure that the genetic divergence of species L. casei and L. rhamnosus based on 16S rRNA gene is very low, which prevents using this site for the design of their differentiating primers. Moreover, the divergence between the other LAB species is also rather low, which can give false cross reactions during PCR set-up.

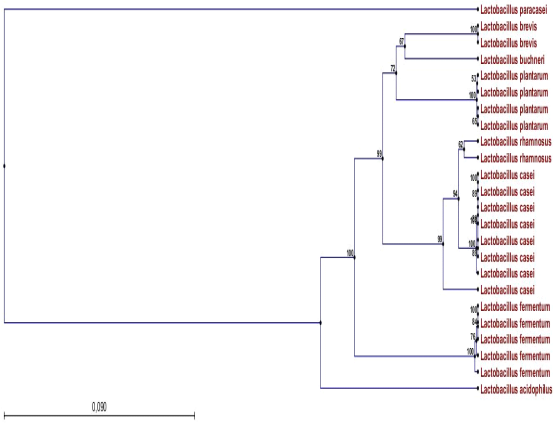

Figure 1a Dendrogram of the genetic divergence based on 16S rRNA gene

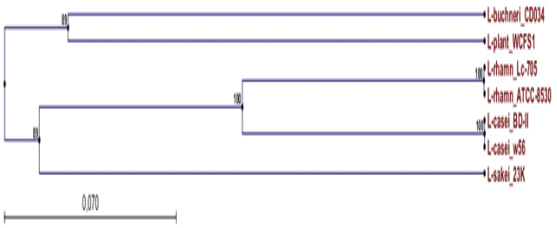

Figure 1b Dendrogram of the genetic divergence based on gyrB gene

Analysis of the genetic distances based on gyrB gene showed that the differences between two closely related species L. casei and L. rhamnosus was significantly higher in this genome site than those in 16S rRNA gene. The divergence of gyrB gene, in general, is 17%. Thus, we decided to use for the selection of the species-specific genome sites the so called housekeeping genes – the genes which are necessary for the maintenance of the basic life functions.
We selected and verified using the on-line BLAST program the primers for identification of closely related species L. casei and L. rhamnosus (Table 1).

Table 1 Developed primers

Primer	Species	5’-3’ sequence
LRh-201F	L.rhamnosus	GTTTGCAACGCGAATTGAAG

LRh-301R	L.rhamnosus	GACCGGTTTTTGCCTGAATG
Lcas-F	L. casei	TCCAGTCTAAAACCGGACGG
Lcas-R	L. casei	TAGGCCACCGGAAACTTTGT

The successful identification of L. casei and L. rhamnosus was achieved with these primers both in the pure culture of microorganisms and directly in the food products manufactured with their use by the direct real-time PCR set-up without isolation of the pure culture and omitting the enrichment stage (Fig.2).

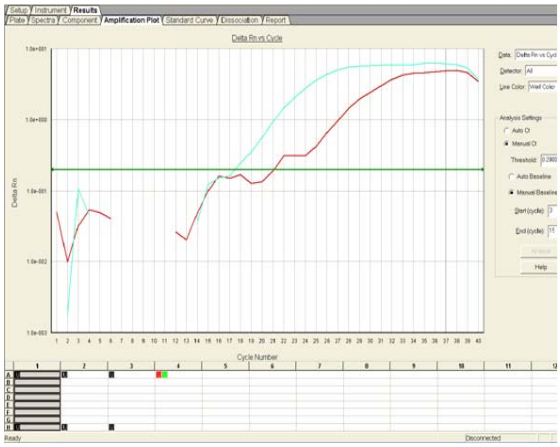

Figure 2 Real-time amplification of DNA isolated from a fermented milk product containing cultures L. casei and L. rhamnosus with the use of species-specific primers for L. casei and L. rhamnosus

With that, it should be noted that the target microorganisms presented in the analyzed subjects on the background of the other LAB species and in some cases in a concentration more than an order of magnitude lower. Use of classical microbiological methods of LAB isolation in this case would not allow to accomplish the set goal.

- CONCLUSION

The species-specific gene sites were revealed on which basis the design of the primers for real-time PCR set-up was possible.
The expedience of use of real-time PCR for the identification of LAB species used in meat industry for their detection both in the composition of a multi-species starter culture and directly in food product was proved.
The possibility of the quantitative assessment of the content of various LAB in analyzing subject was verified.

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