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APPLICATION OF PROBIOTIC *L. CASEI* IN PRODUCTION OF TYPICAL CZECH FERMENTED SAUSAGES

Burdychová R.[1] and Šulcerová H.[1]

[1] Department of Food Technology, Mendel University of Agriculture and Forestry in Brno, Zemědělská 1, 613 00 Brno, Czech Republic. E-mail: burdycho@mendelu.cz

Abstract

The aim of this study was evaluation of possibility to applicate probiotic strain L. casei 431® (Chr. Hansen, Denmark) in the manufacturing process of typical Czech fermented sausages called „Paprikáš". In manufacturing process of those types of sausages, commercial starter culture Pediococcus pentosaceus AS-3/100 (Almi, Austria) is used. Therefore probiotic L. casei was used in combination with this starter culture. Control sausages were prepared using only the starter culture.

L. casei 431® was studied for its ability to produce biogenic amines tyramine and histamine using PCR targeting genes coding for tyrosine- and histidinedecarboxylase and using HPLC. The strain was amine negative. In this study, a rapid working method for selective enumeration and monitoring of survival of probiotic L. casei 431® in fermented sausages was developed. MRS–sorbitol agar was used for selective enumeration of L. casei from probiotic sausages. Molecular analysis of presumptive colonies confirmed their identity as L. casei 431® . In dry sausages, the counts of probiotic L. casei 431® reached concentration of 10^4 CFU/g during fermentation (0–28 days after production) and stayed at relatively constant level during the whole storadge period (28–49 days after production) at 15 °C. Sensory evaluation of dry sausages was performed 28 and 49 days after production. The flavour profiles of the dry sausages produced using probiotic strain L. casei 431® were similar with control sausages. Probiotic culture L. casei LC-01 is suitable for use in manufacture of fermented „Paprikáš"sausages.

Key words: *dry sausages, L. casei 431®, probiotic, HPLC, PCR, MRS–sorbitol agar*

1. INTRODUCTION

Probiotics are defined by the FAO/WHO as live microorganisms which when administered in adequate amounts confer a health benefit on the host. When ingested in sufficient numbers regularly, probiotics are believed to play an important role in the control of host intestinal microbiota and maintenance of its normal state (Fuller, 2000).

During last years, the addition of probiotics into fermented sansages became more popular. The presence of adequate number of live probiotic cells in fermented sausages in the time of consumption represents the first challenge for the development of such probiotic product (Hammes and Hertel, 1998). Rapid detection and identification of various species of probiotic bacteria are often important for monitoring their counts in different types of food.

Some species of probiotic bacteria used in fermented sausages can be difficult to identify when accompanying with other microorganisms used in mixture of starter cultures. Furthermore, fermented sausages have natural high background microbiota. An important parameter in monitoring viable organisms in assessing product quality is the ability to count probiotic bacteria differentially. In order

to assess viability and survival of probiotic bacteria, it is important to have a working method for selective enumeration of these bacteria.

Numbers of working methods for selective enumeration of different probiotic bacteria in foods have been previous proposed by different authors (Beerens, 1990; Shah, 2000; Davidson, 2000; Collins, 1978; Dave and Shah, 1996). These methods are based on culture characteristics of given probiotic often accompanied with starter cultures. Few reports described selective enumeration of *Lactobacillus casei* cells in the presence of starter and yogurt bacteria (Champagne et al., 1997; Ravula and Shah, 1998). Selective enumeration of *L. casei* from probiotic products based on a 15°C incubation temperature and 14 day incubation was studied by Champagne et al. (1997). Ravula and Shah (1998) developed a medium, known as LC agar, for selective enumeration of *L. casei* in fermented milk products. Here we tested the method for selective enumeration of probiotic *L. casei* 431® in fermented meat products.

Combination of microbiology cultivation methods and methods of molecular biology is the best way how to achieve correct qualitative and quantitative *L. casei* 431® probiotic analysis. We present selective method for enumeration of starter and probiotic strains based on different sugar fermentation characteristics. Furthermore, the monitoring of probiotic counst during fermentation and storage period of dry sausages is included.

2. EXPERIMENTAL STUDY

2.1 Materials

L. casei 431® (Chr. Hansen, Danmark) was grown in MRS broth (Noack, France) at 30 °C for 3 days. DNA isolated from this bacteria was used as positive control in PCR analyses.

The commercial starter culture *Pediococcus pentosaceus* AS-3/100 (Almi, Austria) was used in the production of dry sausages.

2.2 Methods

2.2.1 Isolation of DNA and PCR

Standard DNA manipulations were carried out as described in Sambrook et al. (2001) and Ausubel et al. (1994). PCR was carried out in final volume of 25 µl containing 10 ng genomic DNA, 10 pmol of each primer Cas-ITS.L (Massi et al., 2004) and CasII (Walter et al., 2000), 1U *Taq* DNA polymerase and the appropriate amount of Qiagen HotStar Master Mix (Qiagen, Hilden, Germany). DNA samples were first completely denaturated by incubation at 95 °C for 15 min. DNA was amplified in 30 cycles (denaturation at 95 °C for 45 s, primer annealing at 55 °C for 45 s, and elongation at 72 °C for 75 s) using model PTC-150HB thermal cycler (MJ Research, Waltham, MA, USA). In the last amplification cycle, the samples were incubated at 72 °C for 10 min for complete elongation of the final PCR products. After PCR, the amplicons were visualized after 1 % agarose gel electrophoresis (5 V/cm; 60 min) in 0,5 x TBE buffer with ethidium bromide staining (0,5 µg/ml). Detection of genes coding tyrosine and histidinedecarboxylases was carried out using primers TD2/TD5 and HDC3/HDC4 (Coton, et. Al., 2004). The conditions of amplification were the same as above except of temperature of primer annealing which was 52°C.

2.2.2 Detection of tyramine and histamine in growth medium

Cultivation of bacterial strains in decarboxylating medium (Bover-Cid and Holzapfel 1999) and HPLC detection were carried out as described by Burdychova and Dohnal (2007). A PCR detection of genes coding for tyrosinedecarboxylase and histidinedecarboxylase (Coton et al. 2004) was carried

out as described by Burdychova and Komprda (2007). Both, the probiotic *L. casei* 431® and starter culture *P. pentosaceus* AS-3/100 were screened for the production of tyramine and histamine.

2.2.3 Sausages production

Batches of sausages called „Paprikáš" were made using as follows: mixture of frozen pork and pork fat (80 %), beef (20 %), sugar (0,6 % dextrose), 2,5 % nitrate salt (with 0,9% nitrite), E 250, E 316 and spices were added according to traditional recipe. Furthermore, 0,025 % starter and the same amount of probiotic bacteria (*L. Casei* 431®*)* were added. One control batch without probiotic culture was included. The sausages were smoked for 6 days at 26–24 °C and processed in climate chamber in accordance with a traditional four weeks programme (11 – 13 °C, 75 % relative humidity).

2.2.4 Microbiology determination

Sausages were analyzed at the day of production and 14, 28, 35, 42 and 49 days after production to observe probiotic counts. Ten grams of each sample were homogenized in 90 ml of Ringer solution (Noack, France) using Stomacher. A dilution series were made in Ringer solution to obtain 15–300 colony forming units (cfu) per plate. 1 ml of appropriate dilution was plated on MRS–sorbitol agar. This agar consists of a medium that is very similar to MRS agar (deMan, Rogosa and Sharp agar with sorbitol instead of dextrose (20 g/l)). In addition, the pH indicator bromcresol purple (2 g/l, Sigma-Aldrich, Germany) was added. All plates were incubated anaerobically for 3 days at 37 °C. Colonies that were grown on agar plates were differentiated by color, form and edge appearance. The number of colonies corresponding to the square root ot the total count isolated was twice repurified by spreading of the single colonies. Identification to the species level was accomplished by API tests (Biomerieux, France).

2.2.5 Crude cell lysates preparation

Selected colonies were used for preparation of crude cell lysates and for subsequent PCR analysis. The crude cell lysates were prepared by resuspension of a purified single bacterial colony taken from agar medium and resuspended in 10 µl of distilled water. The suspension was centrifuged and washed twice with distilled water. This suspension was then boiled at 100 °C for 10 min before being centrifuged to pellet the cell debris. A total of 1 µl of the crude cell lysate containing genomic DNA was used for subsequent PCR amplification (the same mixture as previous).

2.2.6 Sensory evaluation

The sensory evaluation was performed 28 and 49 days after sausages production (day 0) from dry sausages sloves (h = 3 mm) by a 10-person panel familiar with the sensory evaluation of dry sausages. Determination of appearance on the cut, pleasantness and intensity of flavour and taste, specially of bitter, sweet, sour, metal and salt taste and texture were evaluated, using a continuous unstructurated scale (10 cm) without references.

2.2.7 Statistical analyses

The reported results of chemical, physical and microbiological analysis of sausages made using the probiotic culture were tested with one-way analysis of variance (ANOVA, Statgraphics). The results of sensory evaluation were tested with Tukey's test ($P<0,05$ and $P<0,010$) for multiple comparisons.

3. RESULTS AND DISCUSSION

A number of health benefits have been claimed for probiotic bacteria such as *Lactobacillus casei*. Because of the potential health benefits, this organism is increasingly incorporated into different types of fermented foods. We tested to use probiotic *L. casei* 431® strain (Chr. Hansen, Denmark) together with

the starter culture *P. pentosaceus* AS-3/100 (Almi, Austria) in production of typical czech fermented sausage "Paprikáš" (probiotic Paprikáš).

Growth of L. casei 431® strain on agar plates, microscopy and species-specific PCR

In the first step, *L. casei 431®* strain was used for growth on MRS–sorbitol agar. These cells were grown as convex, medium size colonies that turned the surrounding agar into yellow.These results were in accordance with Chr. Hansen Technical bulletin (2006).

For verification purposes, the number of colonies corresponding to the square root of the total count pf presumptive colonies isolated were picked up and analyzed using microscop. All cells had morfology typical for the genus *Lactobacillus* (Table 1). These colonies were also used for DNA isolation and for species-specific PCR analysis. PCR resulted in amplification of products of expected lenght (188 bp, Fig. 1).

In further experiment, starter culture *P. pentosaceus* AS-3/100 mixed with probiotic strain of *L. casei* were tested for their growth characteristic on MRS–sorbitol selective medium. *L. casei* colonies became yellow, whereas the starter bacteria (pediococci) grown weakly and stayed transparent whitish-blue. Both types of colonies could be well distinquished. It is because *L. casei 431®*ferments sorbitol into acid in contrary to pediococci.

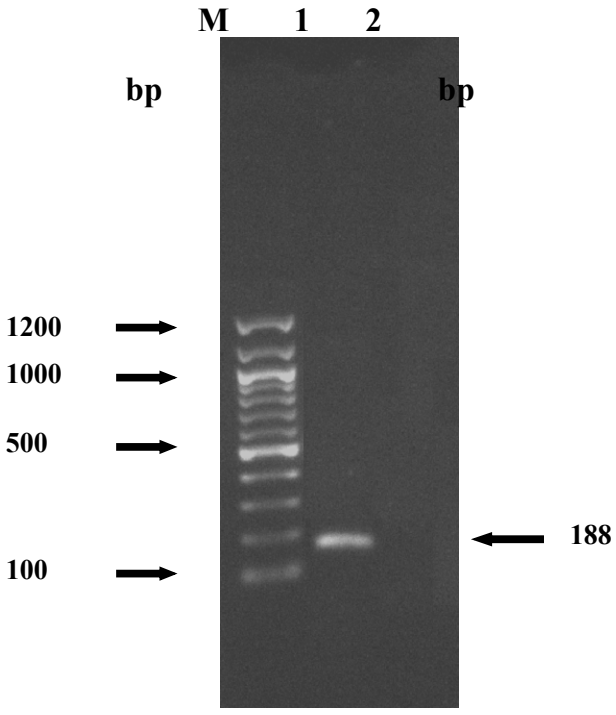


Fig.1: Agarose gel electrophoresis of PCR-based identification of L. casei colonies.
M: 100 bp ladder (New England Biolabs), 1: PCR product amplified from L. casei 431® DNA (1 ng), 2: negative control
One colony PCR for L. casei 431® verification in microbiology analysis of dry sausages

When using MRS–sorbitol agar, some contaminating sorbitol-positive lactic acid bacteria could appear on the agar plates and give also yellow colonies, as *L. casei 431®* cells. At the day of dry sausages production, *L. casei* is clearly distinquishable from the other microorganisms in the sausage since mainly *L. casei* and the starter culture are present in appreciable amounts. Hovewer, later during ripening and storage, other contaminating sorbitol-positive lactic acid bacteria could appear on the agar plates and give yellow colonies. To verify that the counted colonies were indeed *L. casei 431®* a few individual colonies were controlled in a microscope and using biochemical tests and PCR. Results are shown in Table 1.

Table 1: Microscopy, biochemical and PCR identification of presumptive L. casei colonies

Colony number	**Microscopy**	**API tests**	**PCR**
1	sticks	*Lactobacillus* spp.	*L. casei*
2	sticks	*L. lactis*	*L. casei*
3	sticks	*Lactobacillus* spp.	*L. casei*
4	sticks	*L. casei*	*L. casei*
5	sticks	*Lactobacillus* spp.	*L. casei*
6	sticks	*L. casei*	*L. casei*
7	sticks	*Lactobacillus* spp.	*L. casei*
8	sticks	*L. lactis*	*L. casei*

Unfortunatelly it is well known that the biochemical characteristics of bacteria can be changed in food which makes the biochemical identification non accurate (Španová et al., 2001). To preclude the identification problems, a molecular biology identification using PCR were shown to be a good alternative. PCR analysis of single colonies isolated on selective medium confirmed their identity as *L. casei*. According to our experience, PCR was the only specific method which can be used for identification of probiotic *L. casei* strains in fermented sausage „Paprikáš“. Morphology of all cells was the same and API tests were not successful for species differentiation.

Enumeration of Bacteria in sausages

The presence of live probiotic cells of *L. casei* in the amount of 10^4/g at the beginning of ripening and during storage documents survival of probiotic culture in the product (Fig. 2). To provide health benefits, the suggested concentration for probiotic bacteria is 10^6 cfu/g of a product (Shah, 2000) and daily consumption of 100 g of such product is recommended. However, the minimal dose is dependant on several factors such as individual person, probiotic strain and type of food product (Tannock, 2002). Futher experiments will be focused on strategies enhancing viable *L. casei* cells per g of sausages.

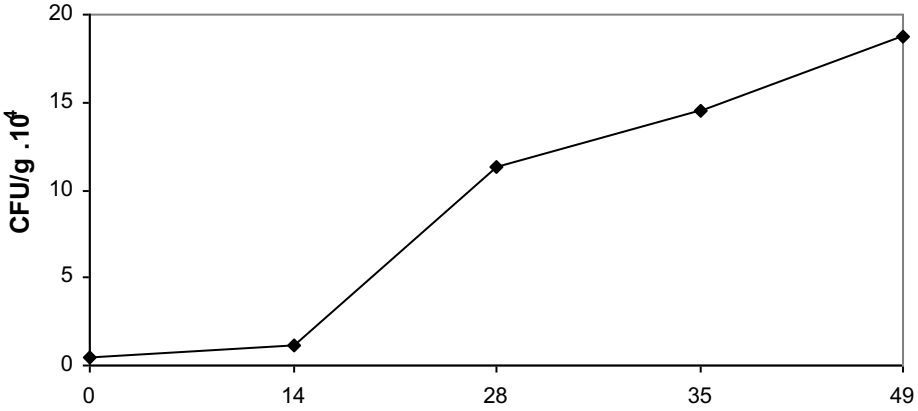


Fig. 2: Changes of probiotic L. casei during fermentation (0–28 days) and storage (28–49 days) of dry sausages „Paprikáš“

Sensory evaluation

Fig. 3 shows the results of descriptive sensosy analysis at the end of ripening (28 days) and at the minimal endurance time (next 21 days). The biggest changes between samples were in descriptors pleasantness of odour, metal taste and sweeteness (Benito *et al.*, 2004). Panel data were analyzed by means of Tukey's test for multiple comparisons. There is no significant differences in parameters between samples (P<0,05 or P<0,010). Fermented sausages are produced as a results of the lactic fermentation of a mixture of comminuted meat mixed with fat, salt, curing agents, sugar and spices and these represent traditional foods of country (Caplice and Fitzgerald, 1999). When producing probiotic meat products, the sensory and technological aspects of the end-products have to be near the traditional products. The results shows that this criterion was realized.

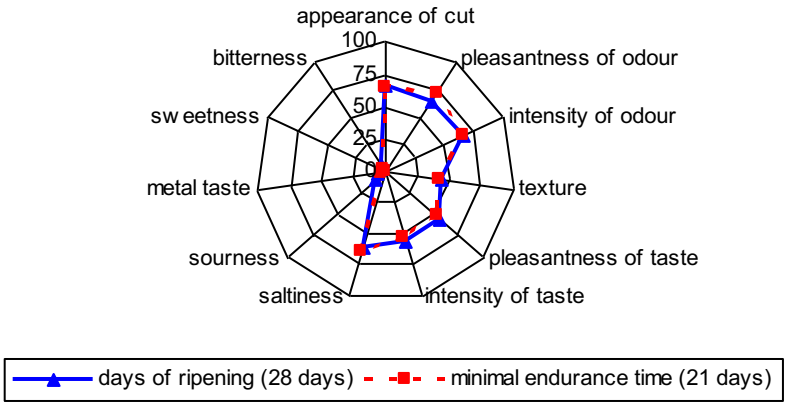


Fig. 3 Sensory profiles of fermented sausage „Paprikáš“ at the end of the ripening (▲) and at minimal andurance time (■)

4. CONCLUSIONS

We have shown that dry sausages are products, which may be suitable carrriers for probiotics into the human gastrointestinal tract (Työppönen et al., 2003). The question still is, if those types of meat products can be called functional foods and consumed daily. When we pass over the fact that probiotics serve as nutraceuticum, fermented sausages are not recommended in healthy nutrition due to the fact that contain high amount of fat (the maximum limit is 50 %) and thanks to it high concentration of saturated fatty acids and cholesterol. Also high concentration of sodium chloride in such type of products (3,5–4%) is in contrary to healthy nutrition and prevention of heart and cardiovascular diseases. Counting the fact that recommended amounts of healthy nutrition strategies are 25 % energy from fat, saturated fat less than 10 %, cholesterol 300 mg or less and sodium 2400 mg or less it, can 1–50 g of such probiotic sausage at normal person with 10 000 KJ daily energy intake be a certain compromise. The best way for healthy nutrition strategy should be combination of milk, meat and vegetable probiotic products and its daily consumation in recommended levels.

REFERENCES

1. Ausubel F.M., Brent R., Kingston R.E., Moore D.D., Seidman J.G., Smith J.A., Struhl K. (1994) Current protocols in molecular biology. *New York: Greene Publishing Associates and Wiley-Interscience*. 350 p. ISBN 0-47150-338-X.

2. Beerens H. (1990) An elective and selective isolation medium for *Bifidobacterium* spp., *Lett. Appl. Microbiol.* 11 (1990), pp. 155–157.

3. Benito M.J., Rodríguez M., Martín A., Aranda E., Córdoba J.J. (2004) Effect of the fungal protease Epg222 on the sensory characteristics of dra fermented sausage „salchichón“ ripened with comercial starter cultures. *Meat Science*, 67, pp. 497-505.

4. Bover-Cid S., Holzapfel W.H. (1999) Improved screening procedure for biogenic amine production by lactic acid bacteria. *Int. J. Food Microbiol*. 53**:** 33–41.

5. Burdychova R., Dohnal V. (2007) The use of HPLC method for determination of microbial tyrosine decarboxylase expression product *Chemical Papers* 101, 907.

6. Burdychova R., Komprda T. (2007) Biogenic amine-formingmicrobial communities in cheese. *FEMS Microbiology Letters 276*, 149.

7. Caplice E., Fitzgerald G.F. (1999) Food fermentations: role ob microorganisms in food production and preservation. *International Journal of Food Microbiology*, 50, pp. 131-149.

8. Collins, E. B. (1978) Enumeration of *Lactobacillus acidophilus* with the agar plate count. *J. Food Prot.* 41:439–442.

9. Coton E., Coton M. (2005) Multiplex PCR for colony direct detection of Gram-positive histamine- and tyramine-producing bacteria. *J. Microbiol. Methods*. 63(3): 296–304.

10. Dave R.I., Shah N.P. (1996) Evaluation of media for selective enumeration of *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactobacillus acidophilus* and *Bifidobacterium* spp. *J. Dairy Sci.* 79:1529–1536.

11. Davidson R.H., Duncan S.E., Hackney C.R., Eigel W.N., Boling J.W. (2000) Probiotic culture survival and implications in fermented frozen yogurt characteristics. *J Dairy Sci.* 83(4):666-73.

12. FAO/WHO (2002). Guidelines for the evaluation of probiotics in food – Joint Food and Agricultural Organization of the United Nations and World Health Organization Working Group Meeting Report, London Ontario, Canada.

13. Fuller R. (1997) Introduction. In Probiotics 2: Applications and practical aspects Edited by: Fuller R., New York, Chapman & Hall, 1-9.

14. Hammes, W. P., Hertel C. (1998) New developments in meat starter culture. *Meat Sci.* 49**:**S125-S138.

15. Hansen Ch. (2006) Method for enumerating *Lactobacillus casei* 431. Technical Bulletin. March 2006.

16. Champagne C. P., Roy D., Lafond A. (1997) Selective enumeration of *Lactobacillus casei* in yoghurt-type fermented milks based on a 15 °C incubation temperature. *Biotechnol. Tech.* 11:567–569.

17. Massi M., Vitali B., Federaci F., Matteuzzi D., Brigidi P. (2004) Identification Metod based on PCR combined with automated ribotyping for tracking probiotic *Lactobacillus* strains colonizing the human gut and vagina. *Journal of Applied Microbiology*, 96, p. 777-786.

18. Ravula R.R., Shah N.P. (1998) Selective enumeration of *Lactobacillus casei* from yogurt and fermented milk drinks. *Biotechnol. Tech.* 12:819–822.

19. Sambrook J. MacCallum P.*,* Russell D. (2001) Molecular cloning: a laboratory manual. 3rd. ed. *New York: Cold Spring Harbor Lab. Press*, 2344 pp. ISBN 0-87969-577-3.

20. Shah N.P. (2000) Probiotic bacteria: Selective enumeration and survival in dairy foods. *J. Dairy Sci.* 83:894–907.

21. Spanova A, Rittich B, Karpiskova R, Cechova L., Skapova D. (2000) PCR identification of Salmonella cells in food and stool samples after immunomagnetic separation. *Bioseparation* 9: 379–384.

22. Tannock G. W. (2002) Probiotics and Prebiotics: Where are We Going? Norwitch: Caister Academic Press. 333 p. ISBN-10: 0-9542464-1-1.

23. Työppönen, S., Petäjä E., Mattila-Sandholm T. (2003) Bioprotectives and probiotics for dry sausages, *International Journal of Food Microbiology* 83, pp. 233–244.

24. Walter J., Tannock G.W., Tilsala-Timisjarvi A., Rodtong S., Loach D. M., Mundro K., Alatossava T. (2000) Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers. *Appl Environ Microbiol* 66:297–303.