Dry-fermented sausage as probiotic carrier food

By Tanja Žugić Petrović, Predrag Ilić, Mirjana Muruzović, Katarina Mladenović, Dragana Stanisavljević and Ljiljana Čomić

The aim of this research is to explore the potential use of *Lactobacillus curvatus* and *Enterococcus faecium* isolated from traditional fermented sausage from Southeast Serbia as probiotics. Analytic criteria for determining the probiotic potential included haemolysis, autoagreggation and coaggregation tests as well as the test for hydrophobicity of natural isolates. The best autoaggregation ability was observed in *Lb. curvatus,* sk2-17 and sk6-5 (73%) while the lowest level of grouping was shown by the isolate sk1-10 (68%), as the coaggregation ability ranged from 34% to 58%. The value of autoaggregation of examined strains of enterococci ranged from 47% to 55.2%, while coaggregation ranged from 31.9% to 45.2%. Haemolytic reaction was not observed in researched isolates. Hydrophobicity was determined by bacterial adherence to hydrocarbons, n-hexadecane, xylene and chloroform. All isolates possessed a high level of hydrophobicity. The results of this study of probiotic characteristics of *Lactobacillus curvatus* and *Enterococcus faecium* indicate that the tested strains can be recommended as good candidates for use in the food industry.

KEYWORDS

>> Hydrophobicit
>> Autoaggregation
>> Coaggregation
>> Haemolysis
>> Probiotic characteristics

Fermented foods are getting more and more attention regarding their positive effects on human health. Spontaneously fermented foods without additives and preservatives are a possible source of microorganisms with probiotic and technological characteristics. These microorganisms could have beneficial effects on human health and also be used as starter cultures in controlled fermentations. In fermented meat products they are protected by fat and surrounding tissue against unfavourable human gastrointestinal (GI) tract conditions, and are able to better manifest their positive probiotic behaviour (Gänzle et al., 1999). Probiotic microorganisms have different characteristics from other microorganisms, as they are able to survive in different parts of the GI tract. These differences are not only within families, but can also be found within the specific species and even strains. Their ability to attach to different places of the intestinal mucosa is especially important (Marteau and Shanahan, 2003). The researches in the area of fermented meat as possible sources of probiotic cultures have been partly disputed because of a trend that meat products are not the most desirable food for human organism due to high content of fat, salt and other ingredients. Addition of probiotics as starter cultures to fermented sausages promotes health benefits of Lactic acid bacteria (LAB) and contributes to the increase of consumption of fermented meat products (Vuyst et al., 2008).

Fermented sausages are a part of the Serbian culture and are not only characterized by unique aroma and taste, but also by prolonged shelf life. Fermented sausages are defined as a mixture of various types of meat with addition of salt, sugar and spices, and they can be smoke-dried, i.e. dried. Quality of these products depends on the fermentation process which defines sensory and nutritive values of the product, and it is in direct relation to microbiota which develops during this process. During spontaneous fermentation of sausages, the growth of bacteria originating from meat or from the production process causes the microbiota to develop spontaneously. Fermented sausage is an autochthonous product from the territory of Southeast Serbia that is produced during autumn and winter. The meat mixture is prepared from cold pork meat with a small quantity of beef and spices. The sausage is smoke-dried for approximately 25 days on cold beech wood smoke, and then dried in air. The sausage dries in the air as long as low air temperatures allow for the preservation of the product. Spontaneous fermentation of this sausage leads to development of characteristic microbiota and sensory characteristics. As the probiotic potential of this product has not been explored so far, the goal of this study was to determine the probiotic characteristics of 30 autochthonous LAB isolates from fermented sausage. This is just a first step that would enable the establishment of logical criteria for screening and selecting microorganisms present in food that show probiotic properties beneficial to humans. This product, in this state, is investigated for the first time to such a high degree. Selection of microorganisms in it is performed for the first time and the possibilities of their application are explored, mainly their probiotic characteristics.

Materials and methods

In the research 30 isolates of LAB isolated from 10 samples of fermented sausage identified by 16s RNA sequencing as *Lactobacillus curvatus* (9 strains) and *Enterococcus faecium* (21 strains) (data not shown) were screened for their probiotic features. All the tests have been performed in triplicate and the results are represented as the average values.

Haemolysis on blood agar plates

Capacity of haemolysis of LAB strains was analyzed by inoculation onto blood agar plates (Torlak, Serbia) with addition of 5% of sheep blood and incubation at the temperature of 37 °C during 24 h. The plates were examined for the existence of light zones, whereby α-haemolysis (insufficiently translucent zones around colonies), β-haemolysis (clear zones around) and γ-haemolysis (without aureole around colonies) indicated a positive haemolytic activity, i.e. pathogenesis of examined strains (Maragkoudakis et al.,2009).

Autoaggregation and coaggregation

Autoaggregation of studied lactobacilli and enterococci, as well as coaggregation with *Escherichia coli* ATCC25922 was monitored using a modified method described by Ocaña et al., (2002). Autoaggregation of examined isolates was monitored in PBS buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, pH 7.5). The cells of overnight culture were settled down by centrifugation at 5000 rpm for 5 minutes, after which cells were washed twice in buffer, and then re-suspended in 4 ml of the same buffer so the number of cells

Received: 21 August 2018 | reviewed: 28 August 2018 | revised: 21 September 2018 | accepted: 24 September 2018

was approximately 10^8 CFU/ml (absorbance of suspension at 600 nm was around 1.0). The suspension was well mixed in Vortex; 100 µl from the suspension surface was transferred into the micro tube with 900 µl PBS of buffer and A_{600} (A_0) was measured. The same procedure was repeated after two hours. Autoaggregation was calculated by applying following equation (Ocaña et al., 2002).

Autoaggregation % = $(A_0 - A_t)/A_0 \times 100$

where At-A_{600nm} after 2 h

For coaggregation, the cells of examined microorganisms and pathogens were prepared in identical way as in the previous method, and then re-suspended in PBS buffer per 2 ml of each suspension of both types of bacteria for which the coaggregation was monitored, and they were mixed well in Vortex, 100 µl from the suspension surface was transferred into the micro tube with 900 µl PBS of buffer and A_{600} (A_i) was measured. Coaggregation was calculated by applying following equation (Ocaña et al., 2002).

Coaggregation %=$(A_i - A_f)/A_i \times 100$

where Af represents the absorbance of supernatant A_{600nm} after 2 h.

Bacterial adhesion to hydrocarbons (hydrophobicity)

The adhesion to the intestinal epithelium is a feature that is very important in the selection of potential probiotic cultures. Bacterial adhesion to hydrocarbons (hydrophobicity) was determined by the modified method was described by Rosenberg et al. (1980). Bacterial strains were grown in De Man, Rogosa and Sharpe (MRS) broth (Torlak, Serbia) at 37 °C for 24 h, after centrifugation at 5000 x g for 15 minutes, the pellets were washed twice with phosphate buffer saline (pH 7.0) and optical densities of the bacteria were measured at 540 nm and adjusted to an optical density of A_{540}=1.0. One ml of bacterial suspension was added to 1 ml of each of the hydrocarbons (xylene, n-hexadecane and chloroform, Sigma/USA) and vortexed vigorously for 30 sec. After phase separation (30 min), the optical density of the aqueous phase was again measured and compared with the initial value. Hydrophobic percentage was calculated based on the equation:

Absorbance% = $(A_0 - At) / A_0 \times 100$

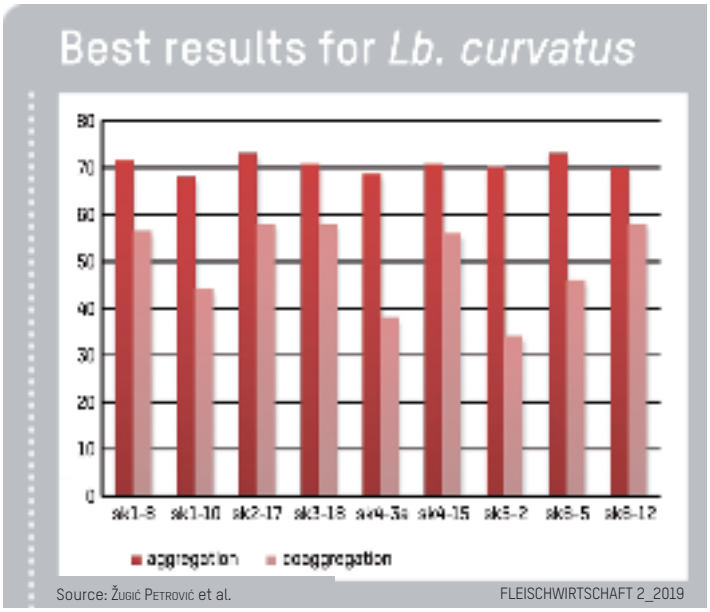

Fig. 1: Aggregation (red bars) and coaggregation (light red bars) of *Lb.curvatus* and *E.coli*
Abb. 1: Aggregation (rote Balken) und Co-Aggregation (hellrote Balken) von *Lb.curvatus* und *E.coli*

Results and discussion

Haemolysis on blood agar

Haemolytic activity was monitored with strains of *Lb. curvatus* and *E.faecium* in order to check for possible pathogenesis of the strains. In the cases of *Lb. curvatus* and *E. faecium* haemolysis on blood agar has not been noticed, which excludes pathogenesis of examined strains. Similar results were also presented in the work by Ledina et al. (2013) who did not noticed haemolysis on blood agar with lactobacilli strains. LAB that were studied by Maragkoudakis et al. (2006) have also shown negative test of haemolysis, so the authors concluded that the examined strains were not pathogenic. The paper of Macovei et al. (2006) showed that positive β-haemolysis in two *E. faecium* strains isolated from food. *Enterococci* must be checked carefully for the presence of haemolytic activity. Although 53.9% of strains isolated in study belong to *enterococcus* genus, fewer than 10% of isolates displayed β-haemolysis, a property that has not been frequently reported for LAB (Miranda et al. 2014).

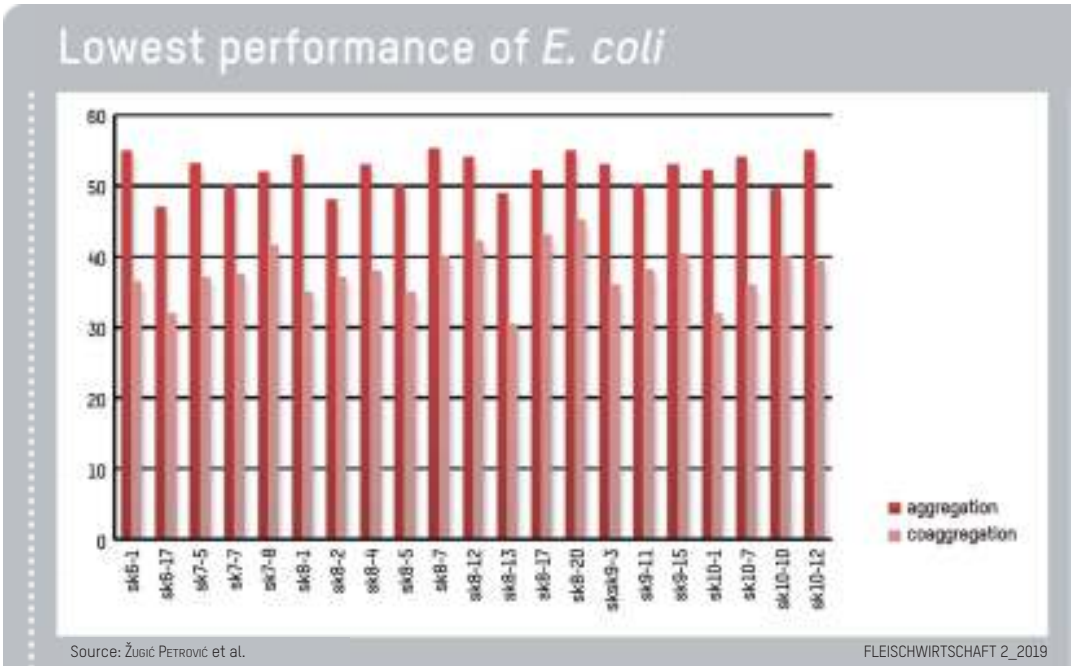

Fig. 2: Aggregation (red bars) and coaggregation (light red bars) of *E. faecium* and *E. coli*
Abb. 2: Aggregation (rote Balken) und Co-Aggregation (hellrote Balken) von *E. faecium* und *E. coli*

The ability of autoaggregation and coaggregation

Intestinal epithelial cells represent the barrier that prevents the interaction of the host with intestinal contents. The grouping of the cells of the same strain in liquid culture represents autoaggregation, while the grouping of different strains is

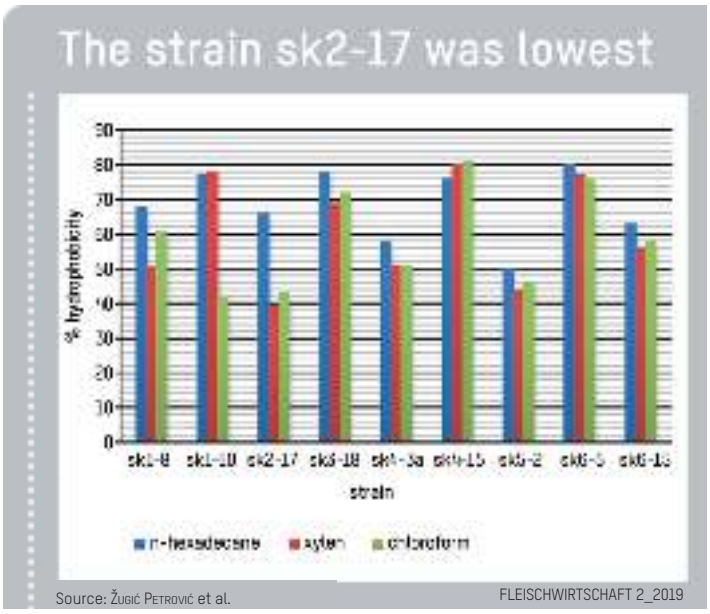

Fig. 3: Percentage of hydrophobicity of *Lb.curvatus*
Abb. 3: Prozentualer Anteil der Hydrophobie an *Lb.curvatus*

coaggregation. The ability of autoaggregation is defined as the measurement of the ability to bind examined cultures to intestinal epithelium in GI tract. Determining this ability *in vitro* is in direct dependence of examined strain, mechanisms of adhesion and conditions of examination. (Del Re et al.,1998). Aggregation is important in choosing probiotics; probiotic strains need to attach to the cell of intestinal epithelium and coaggregate with pathogens to prevent their reproduction (Aslim et al.,2007). The results of the investigation of autoaggregations and coaggregations of examined strains of lactobacilli and *E. coli* strains are shown in Figure 1. Different values of autogroupings were shown by examined isolates of *lactobacilli*, the best autoaggregation is observed for *Lb. curvatus,* sk2-17 and sk6-5, 73% while the lowest level of grouping was shown by the isolate sk1-10 (68%). Sifour et al. (2014) showed that the values of autoaggregation *Lb. curvatus* G6 was 45% and different values for coaggregation of examined *Lb. curvatus* G6 and *Listeria monocytogenes* were observed, *Lb. curvatus* G6 had the higher level of autoaggregation than *Lactobacillus plantarum* ST16Pa6. Ehrmann et al. (2002) indicated high level of coaggregation *Lactobacillus salivarius* with indicator strains of pathogenic *E. coli* CTC 1028, *Salmonella enteritidis* CTC 1039 and *Salmonella typhimurium* CTC 1037. The paper of Kos et al., (2003) implies the significance of coaggregation of LAB strains as potential probiotics with enteropathogenic *E. coli*. Coaggregation with pathogens can represent an important mechanism of defence from infections to human's gastrointestinal and urogenital tract. Some *lactobacilli* are capable of producing certain micro surrounding (autoaggregates and coaggregates) around pathogens with higher concentration of inhibitory substances (Sifour et al., 2014). Coaggregation may be a vital factor in establishing and maintaining microflora (Ramirez-Chavarin et al., 2013).

Todorov et al.,(2009) in their study showed that the value of autoaggregation of examined strain of *enterococci* was 41.34±1.15% while different levels of coaggregation of *Ent.mundtii* ST4V with different strains of *Lactobacillus* and *Listeria* were observed.

Ramirez-Chavarin et al., (2013) indicated high level of coaggregation (23.56% and 30%) *En. faecium* with indicator strains of pathogenic *E. coli* 0139:H26 and *Salmonella parera* IV 011:Z4Z23 (Fig. 2).

Ramirez-Chavarin et al., (2013) concluded that autoaggregative capacity and the ability to coaggregate with pathogenic strains can be used for preliminary selection and identification of probiotic bacteria with potential applications in human and animal systems.

Cell surface hydrophobicity assessment

The hydrophobic nature of the bacterial cells is an important feature of the defining characteristics of probiotic isolates, and depends on the composition and structure of bacterial cell walls, particularly the presence of hydrophobic proteins (Pan et al., 2006). Bacterial cells with strong hydrophobic properties typically form strong interactions with the mucous cells. Hydrophobicity varies among the species and even among strains of the same species (Schär-Zammaretti and Ubbink, 2003). The percentage of hydrophobicity of *Lb. curvatus* isolates from traditionally made fermented sausages ranged from 39% to 81%. All strains that had more than 40% hydrophobicity were considered to be hydrophobic (Boris et al., 1998) (Fig. 3). The hydrophobic nature of a sk2-17 strain to xylene was the lowest in comparison to other isolates, and its value was 39%. While in the case of n-hexadecane and chloroform hydrophobicity value of sk2-17 strain was 66% and 43% respectively. The highest affinity to the solvents used in this study was exhibited by strains sk4-15 and sk6-5, whose hydrophobicity exceeded 75%. Kos et al., (2003) tested the bacterial adhesion to chloroform and ethyl acetate, wherein all of the tested strains showed a stronger affinity to chloroform and the strongest adhesion was in *Lactobacillus acidophilus* M92. The paper of Abdulla et al. (2014) presented the results of hydrophobicity of six lactobacillus strains tested which ranged from 29.5% to 77.4%. Botes et al. (2008) in their work on the adhesion of the probiotic strains emphasize the 50% hydrophobicity in the *Lb. plantarum* 423 strain.

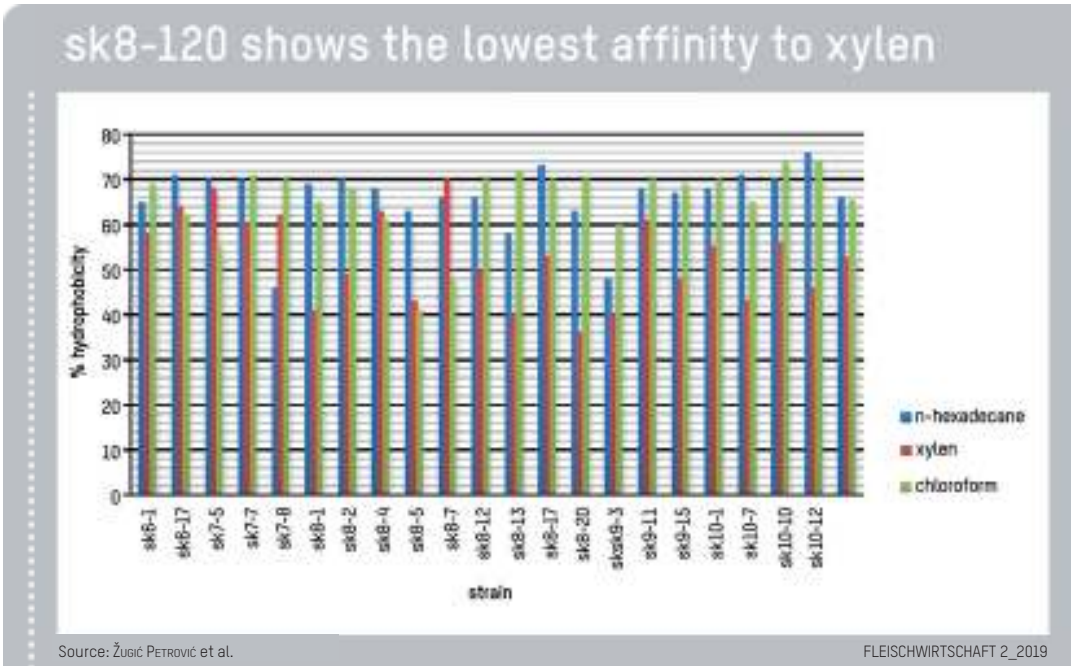

Fig. 4: Percentage of hydrophobicity of *E. faecium*
Abb. 4: Prozentualer Anteil der Hydrophobie von *E. faecium*

In *E. faecium* isolates the lowest hydrophobicity to xylene was exhibited by sk8-20 strain, and its value was 36%. While in the case of n-hexadecane isolate sk7-8 showed the lowest hydrophobicity value, while isolate sk8-5 had a low value hydrophobicity to chloroform, amounting to 41% (Fig. 4). The highest affinity to the solvents that were used in this study showed sk8-17 strain to n-hexadecane, 73%, and sk8-13, whose hydrophobicity to chloroform was 72%. In their study Al Atya et al. (2008) emphasize hydrophobicity of *Enterococcus faecalis* strains isolated from meconium which is over 40%. Also, in the study conducted by Rehaiem et al., (2014) the results showed high mean hydrophobicity (74.06 ± 2:06%) found in *E. faecium* MMRA, and point out that there is little information available regarding the adhesion of the *enterococci* to mucosal cells of men and animals. Hydrophobicity can help with adherence, but is not a prerequisite for colonization by intestinal probiotic bacteria (Ramiah et al., 2007).

Conclusion

The results of this study indicate a high possibility of hydrophobicity shown by the tested strains as well as strong autoaggregation and distortion of the specific coaggregation ability of the strains of the pathogen, as a key feature in the selection of potential probiotic strains. Excluded possibility of haemolysis also confirms the potential of these strains and opens a new *in vivo* research and the confirmation of probiotic character of the tested strains.

References

1. Abdulla, A., T. Abed, A. Saeed (2014): Adhesion, Auto-aggregation and Hydrophobicity of Six *Lactobacillus* strains. Br Microbiol Res J *4 (4),* 381–391. – 2. Al-Atya, A., K. Drider-Hadiouche, K. Ravallec, R. Silvain, A. Vachee, D. Drider (2015): Probiotic potential of *Enterococcus faecalis* strains isolated from meconium. Front Microbiol *6,* 227. – 3. Aslim, B., D. Onal, Y. Beyatli (2007): Factors influencing auto-aggregation and aggregation of *Lactobacillus delbrueckii* subsp. *bulgaricus* isolated from handmade yogurt. J Food Protect *70,* 223–227. – 4. Boris, S., J.E Suarez, F. Vasquez, C. Barbes (1998): Adherence of human vaginal Lactobacilli to vaginal epithelial cells and its interaction with uropathogens. Infect Immu *66,* 1985–1989. – 5. Botes, M., B. Loos, C.A. van Reenen, L.M.T. Dicks (2008): Adhesion of the probiotic strains of *Enterococcus munditii* ST4SA and *Lactobacillus plantarum* 423 to Caco-2 cells under conditons stimulating the intestinal tract, and in the presence of antibiotics and anti-inflammatory medicaments. Arch Microbiol *190,* 573–584. – 6. Del Re, B., A. Busetto, G. Vignola, B. Sgorbati, D. Palenzona (1998): Autoaggregation and adhesion ability in a *Bifidobacterium suis* strain. Lett Appl Microbiol *27,* 307–310. – 7. Ehrmann, M.A., P. Kurzak, J. Bauer, R.F. Vogel (2002): Characterization of lactobacilli towards their use as probiotic adjuncts in poultry. J Appl Microbiol *92 (5),* 966–975. – 8. Gänzle M.G., C. Hertel, J.M. Van der Vossen, W.P. Hammes (1999): Effect of bacteriocin-producing *Lactobacilli* on the survival of *Escherichia coli* and *Listeria* in a dynamic model of the stomach and the small intestine. *Int J Food Microbiol 48,* 21–35. – 9. Kos, B., J. Suskovic, S. Vukovic, M. Simpraga, J., Frece, S. Matosic (2003): Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. J Appl Microbiol *94 (6),* 981–987. – 10. Ledina, T., Z. Mijačević, S. Bulajić, M. Babić (2013): Probiotski status bakterija mlečne kiseline. 176. *Vet J* Republic of Srpska XIII *2,* 176–192. – 11. Macovei, L., L. Zurek (2006): Ecology of antibiotic resistancegenes: characterization of *enterococci* from houseflies collected in food settings. Appl Environ Microbiol *72,* 4028–4035. – 12. Maragkoudakis, P.A. G. Zompoppoulova, C. Mlaisa, G. Kalantzopoulosa, B. Potb, E. Tsakalidou (2006): Probiotic potential of *Lactobacillus* strains isolated from dairy products. Int Dairy J *16,*189–199. – 13. Maragkoudakis, P.A., K.C. Mountzouris, D. Psyrras, S. Cremonese, J. Fischer, M.D. Cantor, E. Tsakalidou (2009): Functional properties of novel protective lactic acid bacteria and application in raw chicken meat against *Listeria monocytogenes* and *Salmonella enteritidis.* Int J Food Microbiol *130,* 219–226. – 14. Marteau, P. and F. Shanahan (2003): Basic aspects and pharmacology of probiotics: an overview of pharmacokinetics, mechanisms of action and side-effects. Best Pract Res Clin Gastroenterol *17 (5),* 725–740. – 15. Miranda, J.M., A. Samuel, C.G. Nebot, A. Cepeda, C.M. Franco, M.P. Calo-Mata (2014): Technological characterization of lactic acid bacteria isolated from beef stored on vacuum-packaged and advanced vacuum skin packaged system. J Food Process Technol *5.6.* – 16. Ocaña, V. and M.E. Nader-Macías (2002): Vaginal lactobacilli: self- and co-aggregation ability. Br J Biomed Sci *59,* 183–190. – 17. Pan, W.H., P.L. Li, Z. Liu (2006): The correlation between surface hydrophobicity and adherence o *Bifidobacterium* strains from centenarians faeces. Anaerobe *12,* 148–152. – 18. Ramiah, K., C.A. Van Reenen, L.M.T. Dicks (2007): Expression of the mucus adhesion genes Mub and MapA, adhesion-like factor EF-Tu and bacteriocin gene plaA of Lactobacillus plantarum 423, monitored with real-time PCR. Int. J. Food Microbiol *116,* 405–409. – 19. Ramirez-Chavarin, M.L., C. Wacher, C.A. Eslava-Campos, M.L. Perez Chabela (2013): Probiotic potential of thermotolerant lactic acid bacteria strains isolated from cooked meat products. Int Food Res J *20 (2),* 991–1000. – 20. Rosenberg, M., G.L. Gutnic, E. Rosenberg (1980): Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett *9,* 29–33. – 21. Rehaiem, A., B.Z. Belgacem, M.R.E. Dovom, B Martinez, A. Rodriguez, M. Manai, N. Perez Guerra (2014): Assessment of potential probiotic properties and multiple bacteriocin encoding-genes of the technological performing strain *Enterococcus faecium* MMRA. Food Control *37,* 343–350. – 22. Schär-Zammaretti, P. and J. Ubbink (2003): The cell wall of lactic acid bacteria: Surface constituents and macromolecular conformations. Biophys J *85,* 4076–4092. – 23. Sifour, M., I. Tayeb, H.O. Haddar, H. Namous, S. Aissaoui (2012): Production and characterization of bacteriocin of *Lactobacillus plantarum* F12 with inhibitory activity against *Listeria monocytogenes* . Online J Sci Technol *2,* 55–61. – 24. Vuyst, L.D., G. Falony, F. Leroy (2008): Probiotics in fermented sausages. Meat Sci *80,* 75–78. – 25. Todorov, S.D., J. von Mollendorff, E. Moelich, N. Muller, C. Witthuhn, L.M.T. Dicks (2009): Probiotic Properties of Bacteriocin from *E. Mundtii*. Food Technol Biotechnol *47 (2),* 178–191.

Zusammenfassung

Trocken-fermentierte Wurst als Lebensmittelträger für Probiotika

Tanja Žugić Petrović, Predrag Ilić und Dragana Stanisavljević – Prokuplje, Serbien; Mirjana Muruzović, Katarina Mladenović und Ljiljana Čomić – Kragujevac, Serbien

Hydrophobizität | Automatische Aggregation | Co-Aggregation | Hämolyse | Probiotische Eigenschaften

Das Ziel dieser Forschung ist es, die mögliche Verwendung von *Lactobacillus curvatus* und *Enterococcus faecium* , die aus traditionellen fermentierten Würsten aus Südostserbien isoliert wurden, als Probiotika zu untersuchen. Die Analyse des probiotischen Potentials umfasste Hämolyse-, Auto-Aggregations- und Co-Aggregationstest sowie den Test auf Hydrophobizität von natürlichen Isolaten. Die beste Auto-Aggregation wurde beobachtet im *Lb. curvatus*, sk2-17 und sk6-5 (73%), während das niedrigste Niveau der Gruppierung durch das Isolat sk1-10 (68%) gezeigt wurde, da die Co-Aggregationsfähigkeit im Bereich von 34% bis 58% lag. Der Wert der Auto-Aggregation von untersuchten Enterokokkenstämmen lag zwischen 47% und 55,2%, während die Co-Aggregation zwischen 31,9% und 45,2% lag. Bei den untersuchten Isolaten wurde keine hämolytische Reaktion beobachtet. Die Hydrophobie wurde durch bakterielle Haftung an Kohlenwasserstoffen, n-Hexadecan, Xylol und Chloroform bestimmt. Alle Isolate wiesen eine hohe Hydrophobie auf. Die Ergebnisse dieser Studie der probiotischen Eigenschaften von *Lactobacillus curvatus* und *Enterococcus faecium* zeigen, dass die getesteten Stämme als gute Kandidaten für die Verwendung in der Lebensmittelindustrie empfohlen werden können

Authors addresses

Tanja Žugić-Petrović (corresponding author: tanja.zugicpetrovic@yahoo.com), Predrag Ilić and Dragana Stanisavljević, Department of Food Science and Technology, College of Agriculture and Food Technology, Ćirila and Metodija 1, 18400 Prokuplje, Serbia; Mirjana Muruzović, Katarina Mladenović, Ljiljana Čomić , Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Radoja Domanovića 12, 34000 Kragujevac, Serbia