Heterofermentative process in dry fermented sausages - a case report

Josef Kameník[1], Marta Dušková[2], Alena Saláková[1], Ondrej Šedo[3]

[1]University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Meat Hygiene and Technology, [2]Department of Milk Hygiene and Technology, Brno, Czech Republic
[3]Core Facility – Proteomics, CEITEC – Central European Institute of Technology, Masaryk University, Brno, Czech Republic

Received September 13, 2012
Accepted February 19, 2013

Abstract

In certain circumstances the fermentation process in dry fermented sausages converts to heterofermentation pathway leading to acetic acid and carbon dioxide beside lactic acid. The study describes two cases of undesirable heterofermentation in dry sausages from two different producers. In the sausage samples (n = 7) the pH value and the content of lactic and acetic acids were measured. Microbial analysis focused on quantitative and qualitative detection of lactic acid bacteria. The acetic acid content varied from 24.28 to 67.41 μmol·g^{-1} dry matter, in the case of samples from the second producer the content of acetic acid (48.45 to 67.41 μmol·g^{-1} dry matter) was higher than the lactic acid content (20.98 to 29.02 μmol·g^{-1} dry matter). The lactobacilli strains from the sausages were assigned to the corresponding species by Matrix-Assisted Laser Desorption-Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) and classified to three groups according to the sugar fermentation pattern (obligately homofermentative, facultatively heterofermentative and obligately heterofermentative) and they caused the heterofermentation process in the samples of dry fermented sausages. The description of the case of heterofermentation process in dry sausages is unique and there is little information about this topic.

Lactic acid, acetic acid, lactobacilli, MALDI-TOF MS

The fermentation process for the production of dry fermented sausages (DFS) is induced predominantly by lactic acid bacteria (Garriga and Aymerich 2007). The genus *Lactobacillus* is applied in the European type products, in particular of the facultative heterofermentative species such as *L. sakei* or *L. curvatus.* The main contribution of lactic acid bacteria (LAB) in the manufacture of DFS resides in the fermentation of carbohydrates, i.e., in the formation of lactic acid. Lactic acid occurs as a final product in the fermentation of carbohydrates (in the case of homofermentation), whereas in heterofermentative fermentation acetic acid, ethanol, and carbon dioxide are also formed (Gounadaki et al. 2009). During the fermentation and ripening of DFS, the effects of lactic acid are shown on the course of pH values (decrease) and on the aroma of the products.

In certain circumstances, however, the heterofermentative process of converting carbohydrates may take place in DFS. The released acetic acid gives the products a severely sour, unpleasant smell and taste. The released carbon dioxide causes porosity on the cut surface. The affected products are practically unsaleable and must be taken out of circulation. The heterofermentative process is therefore completely undesirable for this group of meat products and must be prevented.

The aim of the present study was to describe two cases of undesirable heterofermentative fermentation in a group of DFS from two different manufacturers and highlight the features that accompany these processes.

Address for correspondence:

MVDr. Josef Kameník, CSc, MBA
Department of Meat Hygiene and Technology
Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno
Palackého tř. 1/3, 612 42 Brno, Czech Republic

Phone: +420 604 220 851
E-mail: kamenikj@vfu.cz
http://actavet.vfu.cz/

Materials and Methods

Products analyzed

In November 2011 and June 2012, two groups of DFS were delivered to the Department of Meat Hygiene and Technology at the University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic. In the first case, the delivery contained 3 samples of Paprikáš sausage (samples 1–3) of different ripening period but from a single manufacturer (manufacturer 1). Sample 1 was after 4 weeks of ripening (finished product), sample 2 after 17 days and sample 3 after 10 days from the start of production. In the second case, the delivery contained four kinds of finished product (A, B, C and D from manufacturer 2).

The reason for receiving the samples was due to a completely non-standard fermentation and ripening process; the manufacturers had repeatedly complained about pungent aroma, strongly sour to astringent taste and soft consistency. Through basic sensory examination, each of the cases presented strongly acidic aroma, soft consistency that did not correspond to the age and diameter of the submitted pieces and, last but not least, when sliced, each showed a non-standard cut surface with a mixture greatly lacking any distinctive texture. The findings of the tested samples upon their delivery lead to the carrying out of sensory analysis, physico-chemical and microbiological analyses.

Sensory analysis

The sausages were evaluated by a trained panel consisting of 4 judges selected from the staff members of the Department of Meat Hygiene and Technology. The panellists evaluated various attributes: cut surface appearance, odour, colour, consistency, texture, and taste matrix.

Physico-chemical analysis

Measurements were done in all of the samples and included the setting of pH value, the content of dry matter, lactic acid and acetic acid. The pH values were measured with a Double Pore needle probe (Hamilton, Bonaduz, Switzerland) on a 340i WTW pH-meter (WTW, Weilheim, Germany). Lactic acid (D/L) was determined using an enzymatic test kit (Megazyme, Bray, Ireland). The principle of this method was the following: the enzyme L (+)- or D(−)-lactate dehydrogenase catalysed the oxidation of L (+)- or D(−)-lactate in the presence of nicotinamide adenine dinucleotide (NAD^+), and the product, pyruvate, was trapped by hydrazine, whereas the NADH (reduced form of NAD) was quantified by measuring absorbance at 340 nm (Megazyme 2011a). Acetic acid was determined using an enzymatic test kit (Megazyme). Within the test the acetyl-coenzyme A synthetase in the presence of adenosine- 5'-triphosphate and coenzyme A (CoA) converted acetic acid (acetate) into acetyl-CoA (reaction 1), with the formation of adenosine-5'- monophosphate and pyrophosphate. Citrate synthase in the presence of acetyl-CoA converted oxaloacetate into citrate (reaction 2). The oxaloacetate required in reaction 2 was formed from L-malate and nicotinamide-adenine dinucleotide (NAD+) in the presence of L-malate dehydrogenase (reaction 3). In this reaction, NAD+ was reduced to NADH. This determination was based on the formation of NADH, which was measured by the increase in absorbance at 340 nm (Megazyme 2011b).

The amount of dry matter was determined by method of International Standard (ISO 1442). The result stated for each sample is the mean of three measurements.

Microbiological analyses

Basic processing of the samples was carried out according to the ISO 7218 (2008) standard. The samples were evaluated on the following microbiological indicators: lactic acid bacteria count (ISO 15214, 2000; aerobic cultivation), number of bacteria of the *Enterobacteriaceae* family (ISO 21528-2, 2006), yeast and mold count (ISO 21527-2, 2009), aerobic sporulate (thermal inactivation of a sample of 80 °C/10 min; blood agar, 30 °C, 72 h, aerobic cultivation) and *Lactobacillus* spp. (de Man, Rogosa and Sharpe - MRS agar, 30° C, 72 h, microaerophilic cultivation).

Suspect isolates of lactobacilli with negative oxidase and catalase tests and isolates of aerobic spore-forming bacteria were further identified using a MALDI-TOF mass spectrometer. Isolates were prepared for MALDI-TOF MS analysis according to standard protocol (Freiwald and Sauer 2009). Mass spectra measurements were carried out using an Ultraflex III instrument (Bruker Daltonik, Germany) operated in linear positive ion mode using FlexControl 3.0 software. Mass spectra were processed using Flex Analysis (version 3.0; Bruker Daltonik) and BioTyper software (version 3.0; Bruker Daltonik). The identification results were expressed by BioTyper log(scores) indicating the similarity of the unknown MALDI-TOF MS profile to available database entries. A BioTyper log(score) exceeding 2.3 indicates a highly probable identification at the species level. A BioTyper log(score) between 2.0 and 2.3 means a highly probable identification at the genus level and probable identification at the species level. Only isolates with a log(score) over 2.0 were taken into account.

Results

Through a basic sensory examination, each of the cases presented strongly acidic aroma, soft consistency that did not correspond to the age and diameter of the submitted pieces and, last but not least, when sliced, each showed a non-standard cut surface with a mixture greatly lacking any distinctive texture.

The results of physico-chemical analyses of sausage samples from first and second manufacturer are provided in Tables 1 and 2, respectively.

Microbiological analysis of samples 1–3 from the first manufacturer (Table 3) were aimed primarily on the analysis of LAB. Yeast and mold were detected due to the high

Table 1. Results of physico-chemical analysis of Paprikáš dry sausage samples (manufacturer 1)

Sample	pH	Acetic acid (μmol·g^{-1} dry matter)	D-lactic acid (μmol·g^{-1} dry matter)	L-lactic acid (μmol·g^{-1} dry matter)	Dry matter (%)
1	4.52 ± 0.01	24.28 ± 0.09	53.60 ± 0.35	56.13 ± 0.36	70.25 ± 0.45
2	4.83 ± 0.01	29.69 ± 0.06	37.45 ± 0.07	45.68 ± 0.08	62.74 ± 0.12
3	4.32 ± 0.01	35.68 ± 0.40	49.85 ± 0.44	63.60 ± 0.56	56.96 ± 0.51

The results are given as mean ± standard deviation

Table 2. Results of the physico-chemical analysis of dry sausage samples A–D (manufacturer 2)

Sample	pH	Acetic acid (μmol·g^{-1} dry matter)	D-lactic acid (μmol·g^{-1} dry matter)	L-lactic acid (μmol·g^{-1} dry matter)	Dry matter (%)
A	5.08 ± 0.00	49.58 ± 0.06	29.02 ± 0.03	16.56 ± 0.02	67.2 ± 0.1
B	5.13 ± 0.00	57.80 ± 0.14	25.72 ± 0.06	9.83 ± 0.02	66.1 ± 0.2
C	5.03 ± 0.00	48.45 ± 0.14	20.98 ± 0.06	9.09 ± 0.03	63.6 ± 0.2
D	5.14 ± 0.01	67.41 ± 0.06	25.38 ± 0.02	32.51 ± 0.03	64.9 ± 0.1

The results are given as mean ± standard deviation

Table 3. Results of microbiological analysis on Paprikáš dry sausage samples (manufacturer 1)

Sample	LAB (CFU·g^{-1})	*Lactobacillus* spp.* (CFU·g^{-1})	Yeast (CFU·g^{-1})	Mold (CFU·g^{-1})
1	$5.3.10^8$	$4.4.10^8$	< 10	< 10
2	$5.3.10^8$	$5.9.10^8$	< 10	< 10
3	$1.2.10^9$	$1.0.10^9$	$7.0.10^2$	< 10

LAB - lactic acid bacteria, CFU – colony forming units ;*Sample 1: *Lactobacillus brevis, L. curvatus, L. plantarum, L. alimentarius, L. versmoldensis, L. farciminis;* Sample 2: *L. sakei, L. curvatus;* Sample 3: *L. brevis, L. sakei, L. alimentarius, L. farciminis*

Table 4. Results of microbiological analysis on dry sausage samples A–D (manufacturer 2)

Sample	Aerobic sporulates (CFU·g^{-1})	LAB* (CFU·g^{-1})	*Enterobacteriaceae* (CFU·g^{-1})
A	< 50	$4.5.10^8$	< 10
B	2.10^3	$8.8.10^8$	< 10
C	$4.5.10^2$	1.10^9	< 10
D	< 50	$9.7.10^8$	< 10

*Sample A: *L. brevis, L. farciminis;* Sample B: *L. plantarum;* Sample C: *L. plantarum, L. brevis, L. curvatus / L. fructivorans* (BioTyper log(score) difference between these two species was lower than 0.1), *L. farciminis;* Sample D: *L. plantarum, L. sakei, L. paracasei, L. brevis, L. versmoldensis*

content of paprika and the possible transfer of these groups of micro-organisms from the spices into the meat mixture. Detection of mold and yeast, however, was negative except for sample 3, in which a low yeast content ($7.0.10^2$ CFU·g^{-1}) was detected.

In the case of samples A–D, it was indicated that raw materials of inferior quality were used by the manufacturer. The investigation was therefore focused not only on LAB, but also on the detection of aerobic spore-forming bacteria and bacteria of the family *Enterobacteriaceae.* As evident from Table 4, no representatives of the family *Enterobacteriaceae* were found and detection of aerobic spore-forming bacteria was also low. Sample B showed evidence of the genera *Bacillus subtilis, B. safensis, B. amyloliquefaciens*, while sample C showed only *B. safensis.* The colony forming units (CFU) values in the two groups of samples from manufacturer 1 and 2 corresponded to concentrations ranging from $4.5.10^8$ up to 1.10^9 CFU·g^{-1}.

Discussion

The fermentation process in DFS is controlled by lactic acid bacteria, whose metabolism is homofermentative under optimal conditions (Stahnke and Tjener 2007). During this process only a small amount of acetic acid is produced, which does not affect the taste of the finished product. Fermentation causes a decrease in pH values, which are lowered from an initial 5.8–5.9 to 5.0 or less. However, a pH value of < 4.5 is already considered to be very low, with a negative impact on the taste of the product. In the case of product samples 1–3, we found a relatively large variance in pH values, even when the products were of the same sort (Paprikáš sausage). Sample 3 showed a considerably low pH value (4.32). In contrast, samples A–D had standard pH values that correspond to products of this type, such as Herkules or Poličan sausages (Kameník et al. 2012a).

Different results are evident, however, in the content of both organic acids, i.e., lactic acid and acetic acid. While samples 1–3 contained approximately 83–110 μmol of lactic acid·g^{-1} of dry matter (summarily D- and L- forms), the values found in samples A–D were much lower. They varied from 30 (sample C) to 58 (sample D) μmol·g^{-1} of dry matter. When comparing our results with the literature data, discrepancy can be noted. For example, Girard et al. (1989) tested the effect of pork fat on the process of fermentation and drying of DFS. On day 42, all of the batches showed pH values of around 5.1; lactic acid content fluctuated between 70 and 110 μmol·g^{-1} of dry matter. While analyzing DFS Herkules in our laboratory, we found the highest increase of lactic acid content during the first three days of curing, when concentrations reached 239–300 μmol·g^{-1} of dry matter (Kameník et al. 2012b). During further aging the content no longer increased significantly. In this respect, the resulting lactic acid in the tested samples was lower, especially in products A–D.

On the contrary, in the samples analyzed in this study, the contents of acetic acid were higher. Samples 1–3, showed values of 24–36 μmol·g^{-1} of dry matter, while samples A–D contained practically double those values 50–67 μmol·g^{-1} of dry matter. According to our previous results (Kameník et al. 2012b), the usual finding is up to 20 μmol·g^{-1} of dry matter, even if some of the authors mentioned higher concentrations (Girard et al. 1989). The content of acetic acid in DFS of standard quality, however, is usually 10–20 × lower than lactic acid concentrations. Such a ratio was, however, far from true for the samples we analyzed. In samples 1–3, the lactic acid content was only 2.8 to 4.6 × higher than the acetic acid content. In samples B–D, acetic acid concentrations were even higher than the summary content of both forms of lactic acid.

Katsaras and Leistner (1988) found that lactic acid bacteria in fermented sausages rapidly multiply from the very start of the curing process and throughout the duration of the fermentation process their number remains at values of 7–9 log CFU·g^{-1}, as confirmed

even by our analysis, where the LAB for all of the samples ranged at counts of 8–9 log CFU·g^{-1}.

Species of *Lactobacillus* were isolated from the samples of DFS. By using the method of MALDI-TOF MS, which had already been successfully used for lactobacilli in foods (Dušková et al. 2012), it was possible to classify these species into three groups according to their metabolism of carbohydrates, i.e., obligately homofermentative (*L. farciminis, L. versmoldensis*), facultatively heterofermentative (*L. plantarum, L. sakei, L. curvatus, L. paracasei, L. alimentarius*) and obligately heterofermentative (*L. brevis, L. fructivorans*). Although the dominant species in European DFS are *L. sakei* and *L. curvatus*, it is also possible to isolate other species from the environment of these products (Urso et al. 2006; Drosinos et al. 2007; Lebert et al. 2007; Benito et al. 2008). If lactobacilli with facultatively heterofermentative metabolism have limited access to glucose, or if there is a change in the concentration of oxygen in the environment, there is reversal fermentation from homo- to heterofermentative. The species *L. plantarum*, which under anaerobic conditions produces lactic acid, forms an increased amount of acetic acid and acetoin in an aerobic environment (Pot and Tsakalidou 2009). If environmental conditions change for lactobacilli within a DFS (improper structure of the mix, incorrect gas exchange between the product and the environment, insufficient drying of the product), the lactobacilli may, under normal homofermentative conditions, ferment sugars heterofermentatively. The acetic acid that is created, and possibly other metabolites, then negatively influences the aroma of the products. These unwanted processes occurred in the case of fermentation of the sausage the tested samples came from. The soft consistency indicated improper treatment of the products; it was consistency that did not correspond to the ripening time. The indistinct structure of the cut surface of the sausages also provided evidence of an inappropriate production process. These deviations led to the creation of inappropriate environment in the core of the products and caused a change in the metabolism of the facultatively heterofermentative lactobacilli. As a result, carbohydrates in the mix were converted in the greater part into acetic acid. This lead to a change in taste and, together with the aforementioned imperfections (consistency, structure of the mix), the manufacturer got substandard unsaleable products. The following factors could lead to the changes in sacharide fermentation and to the changes in microbiota of lactic acid bacteria: raw material with high bacterial population, use of starter cultures with low activity, improper structure of the meat mixture within cutting and filling into casings, and inappropriate extrinsic factors within fermentation and ripening.

Acknowledgements

This work was supported by project CEITEC (Central European Institute of Technology) (CZ.1.05/1.1.00/02.0068 funded from the European Regional Development Fund).

References

Benito MJ, Serradilla MJ, Ruiz-Moyano S, Martín A, Peréz-Nevado F, Córdoba MG 2008: Rapid differentiation of lactic acid bacteria from autochthonous fermentation of Iberian dry-fermented sausages. Meat Sci **80**: 656-661

Drosinos EH, Paramithiotis S, Kolovos G, Tsikouras I, Metaxopoulos I 2007: Phenotypic and technological diversity of lactic acid bacteria and staphylococci isolated from traditionally fermented sausages in Southern Greece. Food Microbiol **24**: 260-270

Dušková M, Šedo O, Kšicová K, Zdráhal Z, Karpíšková R 2012: Identification of lactobacilli isolated from food by genotypis methods and MALDI-TOF MS. Int J Food Microbiol **159**: 107-114

Freiwald A, Sauer R 2009: Phylogenetic classification and identification of bacteria by mass spectrometry. Nature Protocols **4**: 732-742

Garriga M, Aymerich T 2007: The microbiology of fermentation and ripening. In: Toldrá, F. (ed.): Handbook of fermented meat and poultry. Blackwell Publishing, Iowa, USA, pp. 555, ISBN: 978-0-8138-1477-3

Girard JP, Bucharles Ch, Berdague JL, Ramihone M 1989. Einfluß ungesättigter Fette auf Abtrocknungs- und

Fermentationsvorgänge von Rohwürsten. (The influence of unsaturated fats on drying and fermentation processes in dry sausages) Fleischwirtschaft **69**: 255-260

Gounadaki A, Skandamis P N, Nychas G.-J. 2009: Fermented meats. In: Fernandes R (ed.): Microbiology handbook. Meat products. Leatherhead Food International Ltd., Surrey, UK. pp. 297. ISBN: 978-1-905224-66-1

International Standard ISO 15214 (2000): Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of mesophilic lactic acid bacteria – Colony-count technique at 30 °C. Czech office for standards, metrology and testing, 12 p.

International Standard ISO 21527-2 (2009): Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of yeasts and moulds – Part 2: Colony count technique in products with water activity less than or equal to 0.95. Czech office for standards, metrology and testing, 16 p.

International Standard ISO 21528-2 (2006): Microbiology of food and animal feeding stuffs - Horizontal methods for the detection and enumeration of Enterobacteriaceae. Part 2, Colony count method. Czech office for standards, metrology and testing, 12 p.

International Standard ISO 7218 (2008): Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations. Czech office for standards, metrology and testing, 68 pp.

International Standard ISO 1442 (1997): Meat and meat products: Determination of moisture content (Reference method). Czech office for standards, metrology and testing, 4 p.

Kameník J, Saláková A, Bořilová G, Pavlík Z, Standarová E, Steinhauser L 2012a: Effect of storage temperature on the quality of dry fermented sausage Poličan. Czech J Food Sci **30**: 293-301

Kameník J, Steinhauserová P, Saláková A, Pavlík Z, Bořilová G, Steinhauser L, Ruprich J 2012b: The influence of various pork fat types on the ripening and characteristics of dry fermented sausages. Czech J Food Sci, in press.

Katsaras K, Leistner L 1988: Topographie der Bakterien in der Rohwurst (Topography of bacteria in fermented sausage). Fleischwirtschaft **68**: 1295-1298

Lebert I, Leroy S, Talon R 2007: Microorganisms in traditional fermented meats. In: Toldrá F (ed.): Handbook of Fermented Meat and Poultry. Blackwell Publishing, Iowa, USA, pp. 555, ISBN: 978-0-8138-1477-3

Megazyme: Acetic acid assay procedure K-ACET 02/11. Megazyme International Ireland Limited, 2011b, 12 p.

Megazyme: D-Lactic acid and L-Lactic acid assay procedure K-DLATE 05/11. Megazyme International Ireland Limited, 2011a, 22 p.

Pot B, Tsakalidou E 2009: Taxonomy and metabolism of *Lactobacillus*. In: Ljungh Å and Wadström T (ed): *Lactobacillus* Molecular Biology. From Genomics to Probiotics. Caister Academic Press, Norfolk, UK, 205 p. ISBN: 978-1-904455-41-7

Stahnke L H, Tjener K 2007: Influence of processing parameters on cultures performance; pp. 187-194; In: Toldrá F (ed.): Handbook of Fermented Meat and Poultry. Blackwell Publishing, Iowa, USA, 555 p., ISBN: 978-0-8138-1477-3

Urso R, Comi G, Cocolin L 2006: Ecology of lactic acid bacteria in Italian fermented sausages: isolation, identification and molecular characterization. Syst Appl Microbiol **29**: 671-680