

# Rapid identification of bacteria in positive blood culture by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

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**Abstract** Blood culture is probably the most significant specimen used for the diagnosis of bacterial infections, especially for bloodstream infections. In the present study, we compared the resin-containing BD BACTEC™ Plus-Aerobic (Becton Dickinson), non-charcoal-containing BacT/Alert® SA (bioMérieux), and charcoal-containing BacT/Alert® FA (bioMérieux) blood culture bottles with direct identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). A total of 103 bacterial isolates, from clinical blood cultures, representing the most frequent 13 genera and 24 species were examined. Bacteria were extracted from positive blood culture broth by density centrifugation and then subjected to identification by MALDI-TOF MS using two different volumes and chemical treatments. Overall, correct identification by MALDI-TOF MS was obtained for the BD BACTEC™ Plus-Aerobic, BacT/Alert® SA, and BacT/Alert® FA blood culture bottles in 72%, 45.6%, and 23%, respectively, for Gram-negative bacteria in 86.6%, 69.2%, and 47.1%, respectively, and for Gram-positive bacteria in 60.0%, 28.8%, and 5.4%, respectively. The lack of identification was observed mainly with viridans streptococci. Depending on the blood culture bottles used in routine diagnostic procedures and

the protocol for bacterial preparation, the applied MALDI-TOF MS represents an efficient and rapid method for direct bacterial identification.

## Introduction

With the long delays that exist between blood culture sampling and the availability of results, the usefulness of these cultures in infectious disease emergencies has recently been questioned. Among methods that allow quicker bacterial identification from growing colonies, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been shown to accurately identify bacteria that are routinely isolated in a clinical microbiology laboratory. MALDI-TOF MS is a fast, reliable, and cost-effective technique which has the potential to replace and/or complement conventional phenotype identification for most bacterial strains isolated in microbiology laboratories. It has been shown to identify bacterial isolates in 93.2% at the species level and 98.5% at the genus level [1]. In most cases, colonies can be identified to the species level after direct deposition on the target plate. When identification of the species cannot be achieved, an extraction may be necessary. Discordant results are often due to systematic database-related taxonomical differences that can be reduced in the future with an expanding database.

MALDI-TOF MS has also been recently introduced as a new method for the direct identification of bacteria from positive blood culture bottles. Previous studies have mostly used the resin- and non-resin-containing Bactec™ systems (Becton Dickinson) Plus-Aerobic and Standard-Aerobic blood culture bottles [2–8], that demonstrated overall species identification rates of 57% to 95%. Blood culture

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media are supplemented either with resins (Becton Dickinson) or activated charcoal (bioMérieux) to bind antimicrobial molecules and, thereby, enhance the bacterial detection rate by the automates. Recently, a descriptive study demonstrated a low sensitivity for the direct identification from positive non-charcoal-containing BacT/Alert<sup>®</sup> FA culture bottles (bioMérieux) by MALDI-TOF MS of 32% [9]. A direct comparison of different types of culture bottles, including the non-charcoal-containing BacT/Alert<sup>®</sup> SA bottles and the non-resin-containing bottle Bactec<sup>™</sup> Standard Aerobic, showed an identification rate of 61.5% and 76.2%, respectively [10]. However, the number of isolates tested in the latter two studies was low.

In the studies, a wide range of pellet preparation protocols were applied, ranging from two to three centrifugation steps, with an overall centrifugation time of a minimum 5.5 min to a maximum 55 min, and potentially included a serum separator tube and an additional erythrocyte lysis step [2–10].

Most published MALDI-TOF analyses of bacterial colony identification cultured on agar plates, and of bacteria enriched from positive blood cultures, are performed on the Bruker and not Shimadzu system [2–10]. One report comparing the conventional phenotype identification of bacteria grown on agar plates with MALDI-TOF MS from Bruker and Shimadzu showed a significantly higher identification rate with the Bruker system (94.4%) compared to the Shimadzu system (88.8%). However, the proportion of correct high-confidence identifications, the rate of MALDI-TOF MS identification in case of disagreement of the phenotype and spectroscopy results, and the rate of correct MALDI-TOF MS identification in cases of unresolved phenotype identification was higher for the Shimadzu compared to the Bruker system [11]. Thus, the Shimadzu system appears to be an equal alternative to the Bruker MS system.

As either resin- or charcoal-containing blood culture bottles are mostly used in routine blood culture diagnostics, in the present study, we attempted to identify bacteria directly from blood culture bottles, comparing the resin-containing medium BD BACTEC<sup>™</sup> Plus-Aerobic with the charcoal-containing blood culture medium BacT/Alert<sup>®</sup> FA. As preliminary results indicated a low sensitivity of direct identification from BacT/Alert<sup>®</sup> FA, we examined the effect of the charcoal by including the non-charcoal-containing medium BacT/Alert<sup>®</sup> SA in the study. MALDI-TOF MS was performed on a Shimadzu system.

## Materials and methods

### Setting

The microbiology and infectious disease department of the Medical Care Centre, Dr. Stein and Colleagues is located in

mid-western Germany within the Euro region. It receives about 80,000 blood culture bottles per year from hospitals and rehabilitation centers that are located within a 1-h drive of the Medical Care Centre.

### Blood culture

One hundred and three bacterial strains identified by VITEK 2 with a statistical probability  $\geq 99\%$  and representing the routine repertoire of bacterial microorganisms in blood culture from clinical patients during the study period from September to December 2009 with a frequency of  $\geq 1\%$  of positive blood cultures were inoculated into BD BACTEC<sup>™</sup> Plus-Aerobic (Becton Dickinson), BacT/Alert<sup>®</sup> SA (bioMérieux), and BacT/Alert<sup>®</sup> FA (bioMérieux) with a 0.5 McFarland suspension (150  $\mu$ l in sterile 0.45% NaCl solution), together with 5 ml of sterile horse blood (Thermo Scientific). The blood culture bottles were incubated at  $36^\circ\text{C} \pm 0.5^\circ\text{C}$  until growth was positively detectable by the automate. As controls, blood culture broth from the respective bottles was taken aseptically, sub-cultured on control agar plates, incubated in an aerobic atmosphere at  $36^\circ\text{C} \pm 2^\circ\text{C}$  with 5%  $\text{CO}_2$  for 18 h to 24 h, and then submitted again for identification testing by the VITEK 2 system.

### Mass spectrometry

A quantity of 1.5 ml of blood culture broth from positive bottles was transferred into Vacutainer SST II (Becton Dickinson). After centrifuging at 3,300 rpm at room temperature for 10 min, the supernatant was discarded. The pellet on the surface of the gel was eluted with 1.3 ml of sterile water and centrifuged for 1 min at 13,000 rpm. The supernatant was pipetted off and a sample of the pellet was spotted with sterile pipette tips in quadruplicates onto a FlexiMass<sup>™</sup> MALDI target (Shimadzu Biotech). Deposits 1 to 4 were applied as follows:

Deposit 1: 0.5  $\mu$ l of the sample was covered with 0.5  $\mu$ l of 20 mg 2,5-dihydroxybenzoic acid (DHB; AnagnosTec).

Deposit 2: 0.5  $\mu$ l of the sample was covered with 0.5  $\mu$ l of formic acid (FA: 25%; AnagnosTec). After drying, the sample was covered with DHB.

Deposit 3: 0.2  $\mu$ l of the sample was applied to the target plate and chemically treated as in Deposit 1.

Deposit 4: 0.2  $\mu$ l of the sample was applied and chemically treated as in Deposit 2.

The matrix sample was crystallized by air drying at room temperature for 5 min. Measurements were performed with a Shimadzu Biotech AXIMA Assurance<sup>™</sup> mass spectrometer equipped with a 337-nm nitrogen laser and a maximum

pulse rate of 50 Hz (50 laser shots per second) at 220 volts. Spectra were recorded in positive linear mode in the range of 2 to 20 kDa. Each spectrum was obtained after 500 shots. The optimum number of peaks was dependent on the bacterial species and ranged between 50 and 200. Data were automatically acquired using the Shimadzu Biotech Launchpad 2.8 and analyzed by Saramis software, release 3.3.2 (37.811 ReferenceSpectra/2.876 SuperSpectra).

For MALDI-TOF analysis, we considered an isolate to be correctly identified when a minimum of 800 points were matched (SuperSpectrum™). If identification was not achieved according to SuperSpectrum™, the isolates were, nevertheless, considered to be identified when the top 10 proposed bacteria were identical. In case of a discrepancy in identification by VITEK 2 and MALDI-TOF MS (none or different identification to the genus or species level by MALDI-TOF MS) of pellets originating from BACTEC™ Plus-Aerobic bottles and treated with DHB alone, 16S rRNA sequencing was performed. Under these criteria, 16S rRNA sequencing was necessary for 18 isolates (one *Enterobacter cloacae*, one *Escherichia coli*, one *Proteus mirabilis*, one *Enterococcus faecium*, one *Staphylococcus capitis*, three *S. epidermidis*, three *S. haemolyticus*, one *S. hominis*, one *S. saprophyticus*, one *Streptococcus agalactiae*, one *S. anginosus*, one *S. gallolyticus*, one *S. pyogenes*, and one *S. parasanguis*).

#### Preparation of samples for identification by VITEK 2

From a portion of the pellet and a colony of the agar plate (control), a suspension was prepared of 0.5–0.63 McFarland for identification using the VITEK 2 XL apparatus (bioMérieux) according to the manufacturer's instructions.

#### 16S rRNA sequencing and sequence analysis

Bacterial DNA was extracted with the DNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The DNA region coding for 16S ribosomal RNA was amplified by using universal primers 16S-27f (AGAGTTT GATCMTGGCTCAG) and 16S-907r (CCGTCAATC MTTTRAGTTT), which are targeted to universally conserved regions (<http://rdna4.ridom.de/static/primer.html>). Polymerase chain reaction (PCR) assays were performed in an MJ PTC-200 Thermal Cycler (MJ Research). The 50- $\mu$ l reaction mixture includes 1  $\mu$ M of each primer and 5  $\mu$ l of genomic DNA in 1 $\times$ HotStarTaq Master Mix (QIAGEN). Prior to amplification, DNA was denatured for 15 min at 95°C. Amplification was performed with 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension step of 10 min at 72°C. The PCR products were purified with the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions. Se-

quencing of the PCR products was performed with the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions. For sequencing, the primers 16S-27f and 16S-519r (GWA TTACCGCGGCKGCTG) (<http://rdna4.ridom.de/static/primer.html>) were used.

Sequencing products were purified with the DyeEx 2.0 Spin Kit (QIAGEN) according to the manufacturer's instructions and analyzed using an automatic DNA sequencer (ABI Prism 3130 Genetic Analyzer; Applied Biosystems). The obtained sequences were queried against both local and public databases.

## Results

Of the three blood culture bottles and four methods tested, the BD BACTEC™ Plus-Aerobic system provided the highest overall identification rates (average 71.1%, minimum 57.3%, maximum 82.5%; Tables 1 and 2). The best overall identification rate was achieved by the application of 0.5  $\mu$ l of pellet and covered by DHB alone (82.5%). The identification rate of the BacT/Alert® FA blood culture medium was low, with an average of 22.8% (minimum 14.6%, maximum 35.9%). The detection rate of BacT/Alert® SA blood culture bottles was an average of 45.6% (minimum 41.7%, maximum 48.5%), and it did not show a preferred method for pellet application.

Regardless of the blood culture system used, the identification rates for Gram-negative bacteria was higher than for Gram-positive strains (Gram-negative: BD BACTEC™ Plus-Aerobic 86.6%, BacT/Alert® SA 69.2%, BacT/Alert® FA 47.1%; Gram-positive: BD BACTEC™ Plus-Aerobic 60.0%, BacT/Alert® SA 28.8%, BacT/Alert® FA 5.4%; Tables 1 and 2).

Gram-negative bacteria were identified correctly from BD BACTEC™ Plus-Aerobic bottles in 97.7% of the tested isolates when 0.5  $\mu$ l of the pellet applied to the matrix was covered with DHB plus FA, and 93.0% when 0.5  $\mu$ l of the pellet was crystallized with DHB alone (Tables 1 and 2). With both methods, incorrectly identified isolated were not observed. Two isolates not identified to the species level were correctly identified to the genus level (Table 1). Gram-negative bacteria were identified correctly from BD BACTEC™ Plus-Aerobic bottles in 81.4% of the tested isolates when 0.2  $\mu$ l of the pellet applied to the matrix was covered with DHB plus FA, and 74.4% when 0.2  $\mu$ l of the pellet was crystallized with DHB alone (Tables 1 and 2). From the isolates not successfully identified to the species level, one isolate was correctly identified to the genus level (Table 1). There was no misidentification of Gram-negative isolates from BD BACTEC™ Plus-Aerobic. Also, for BacT/Alert® SA and BacT/Alert® FA, identification was



**Table 2** Ranking list of the results of the species identification by MALDI-TOF MS, the blood culture broth, and the protocol used for the deposit treatment

Blood culture bottle	Sample volume ( $\mu$ l)	Reagent	Identification rate (%)
All bacteria			
BD BACTEC™ Plus-Aerobic	0.5	DHB	82.5
BD BACTEC™ Plus-Aerobic	0.5	DHB+FA	76.7
BD BACTEC™ Plus-Aerobic	0.2	DHB+FA	68.0
BD BACTEC™ Plus-Aerobic	0.2	DHB	57.3
BacT/Alert® SA	0.2	DHB+FA	48.5
BacT/Alert® SA	0.5	DHB+FA	46.6
BacT/Alert® SA	0.5	DHB	45.6
BacT/Alert® SA	0.2	DHB	41.7
BacT/Alert® FA	0.5	DHB+FA	35.9
BacT/Alert® FA	0.2	DHB+FA	22.3
BacT/Alert® FA	0.5	DHB	18.4
BacT/Alert® FA	0.2	DHB	14.6
Gram-negative bacilli			
BD BACTEC™ Plus-Aerobic	0.5	DHB+FA	97.7
BD BACTEC™ Plus-Aerobic	0.5	DHB	93.0
BD BACTEC™ Plus-Aerobic	0.2	DHB+FA	81.4
BacT/Alert® SA	0.5	DHB+FA	81.4
BD BACTEC™ Plus-Aerobic	0.2	DHB	74.4
BacT/Alert® SA	0.2	DHB+FA	69.8
BacT/Alert® SA	0.5	DHB	67.4
BacT/Alert® FA	0.5	DHB+FA	67.4
BacT/Alert® SA	0.2	DHB	58.1
BacT/Alert® FA	0.2	DHB+FA	48.8
BacT/Alert® FA	0.5	DHB	41.9
BacT/Alert® FA	0.2	DHB	30.2
Gram-positive cocci			
BD BACTEC™ Plus-Aerobic	0.5	DHB	75.0
BD BACTEC™ Plus-Aerobic	0.5	DHB+FA	61.7
BD BACTEC™ Plus-Aerobic	0.2	DHB+FA	58.3
BD BACTEC™ Plus-Aerobic	0.2	DHB	45.0
BacT/Alert® SA	0.2	DHB+FA	33.3
BacT/Alert® SA	0.5	DHB	30.0
BacT/Alert® SA	0.2	DHB	30.0
BacT/Alert® SA	0.5	DHB+FA	21.7
BacT/Alert® FA	0.5	DHB+FA	13.3
BacT/Alert® FA	0.2	DHB	3.3
BacT/Alert® FA	0.2	DHB+FA	3.3
BacT/Alert® FA	0.5	DHB	1.7

highest when 0.5  $\mu$ l of pellet was covered with both DHB plus FA (81.4% and 67.4%, respectively). Two Gram-negative isolates from 0.2- $\mu$ l pellets of BacT/Alert® SA bottles were misidentified as *Nocardia* spp. and *Clostridium difficile*. No misidentification was observed in strains isolated from BacT/Alert® FA bottles (Table 1). Identification by MALDI-TOF MS to the species level of 0.5  $\mu$ l of

pellet from all three blood culture bottles treated with DHB alone or DHB plus formic acid, was better than of 0.2  $\mu$ l of pellet (BD BACTEC™ Plus-Aerobic 95.3% versus 77.9%, BacT/Alert® SA 74.4% versus 64.0%, BacT/Alert® FA 54.7% versus 39.5%, respectively). Identification of the 0.5- $\mu$ l pellet extracted from BD BACTEC™ Plus-Aerobic bottles and covered with DHB plus FA failed in the case of

one *Citrobacter freundii* isolate (Table 1). When the 0.5- $\mu$ l pellet was treated with DHB alone, identification was unsuccessful in 1 out of 4 *E. cloacae* isolates (which provided correct genus identification), 1 out of 15 *E. coli* isolates, and 1 out of 3 *P. mirabilis* isolates (where correct genus identification was detected). All of the tested *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *K. oxytoca*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Serratia marcescens*, and *Stenotrophomonas maltophilia* were correctly identified.

Gram-positive bacteria were identified correctly from BD BACTEC™ Plus-Aerobic bottles in 75.0% of the tested isolates when 0.5  $\mu$ l of the pellet applied to the matrix was covered with DHB alone, and in 61.7% when treated with DHB plus FA (Tables 1 and 2). The identification of 0.2  $\mu$ l of pellets incubated with DHB alone and DHB plus formic acid was 45.0% and 58.3%, respectively. In contrast, independent of the volume of pellet and the treatment applied, the identification rate of Gram-positive bacteria enriched from BacT/Alert® SA and BacT/Alert® FA bottles was significantly lower, ranging from a minimum of 1.7% to a maximum of 33.3% (average 17.1%). Identification of the 0.5- $\mu$ l pellet from BD BACTEC™ Plus-Aerobic bottles covered with DHB alone failed in 1 out of 4 *E. faecium*, 1 out of 2 *S. capitis*, 3 out of 16 *S. epidermidis*, and 3 out of 4 *S. haemolyticus* tested isolates (Table 1). *S. hominis*, *S. saprophyticus*, *S. agalactiae*, *S. anginosus*, *S. gallolyticus*, *S. pyogenes*, and *S. parasanguis*, of which one isolate was examined, were not identified by MALDI-TOF MS from BD BACTEC™ Plus-Aerobic bottles. When identification of the species was not obtained, the correct identification of the genus was obtained in two isolates from BD BACTEC™ Plus-Aerobic bottles (Table 1). However, *S. agalactiae* from a BD BACTEC™ Plus-Aerobic bottle was misidentified as *S. epidermidis*. From the strains not successfully identified from BacT/Alert® SA and BacT/Alert® FA bottles, six were misidentified as *Nocardia* spp. (twice), *N. otitidiscaviarum*, *C. difficile* (three times), and *K. pneumonia* (Table 1).

In summary, the highest identification rate without misidentification was achieved from 0.5- $\mu$ l pellets originating from BD BACTEC™ Plus-Aerobic bottles.

## Discussion

The bacterial repertoire analyzed in this study, of 35% enterobacteria, 7% non-fermenting bacteria, 24% coagulase-negative bacteria, 18% *S. aureus*, 12% enterococci, and 5% streptococci, reflects the average distribution of bacteria obtained from blood cultures from the complete spectrum of health care providers. It is similar to the distribution of bacterial isolates examined in some

other published reports, describing the applicability of MALDI-TOF MS of the Bruker system from resin-containing BD BACTEC™ Plus-Aerobic [3, 7] or non-resin-containing BD BACTEC™ Standard-Aerobic blood cultures bottles [5].

This study with the MALDI-TOF MS system from Shimadzu shows that, regardless of the protocol used, the overall identification and identification of Gram-negative and Gram-positive bacteria by MALDI-TOF MS was higher from the BD BACTEC™ Plus-Aerobic than from the non-charcoal-containing BacT/Alert® SA and the charcoal-containing BacT/Alert® FA. Bacteria extracted from the BacT/Alert® FA broth showed the lowest identification rate. It confirms the assumptions from small studies with BacT/Alert® SA blood culture bottles and the Bruker system that the identification of bacteria grown in BacT/Alert® from bioMérieux is low [9, 10] and less than satisfactory.

To examine the effect of charcoal on the low MALDI-TOF MS identification rate, we tested various protocols, including different, longer, and more centrifuging steps in preliminary studies. However, elimination of the charcoal improved the results only insignificantly (not shown). The bacterial identification rate from BacT/Alert® SA bottles was not significantly higher than from BacT/Alert® FA, but was significantly lower than from BD BACTEC™ Plus-Aerobic blood culture bottles. This comparison implies that, besides the charcoal, other substances in the BacT/Alert® bottles inhibit spectroscopy measurements.

Here, we present a short protocol of bacterial enrichment with two centrifuging steps that only requires 11 min. The first separation step is performed with a separator gel. An additional step for erythrocyte lysis is not necessary. Interestingly, amongst the reported studies of the rapid identification of bacteria in positive blood culture broths by MALDI-TOF MS, those containing a centrifuging step with a serum separator provided the highest identification rates of 90% and higher, regardless of the number of centrifuging steps, the length of centrifuge time, and additional lysis steps applied [5, 8].

The overall identification rate and the identification rate of Gram-negative and Gram-positive bacteria isolated directly from BD BACTEC™ Plus-Aerobic blood culture bottles and tested by a MALDI-TOF Shimadzu Biotech AXIMA Assurance™ mass spectrometer was equal to or higher than previously described for the Bruker system [2–11]. These results imply that the Shimadzu system does, indeed, offer an alternative to the Bruker system.

The inoculation and treatment of bacterial pellets on the FlexiMass™ MALDI target was tested by four different approaches. Application of 0.5  $\mu$ l of bacteria and sequential treatment with DHB plus FA resulted in the highest

identification rates for all Gram-negative bacteria extracted from BD BACTEC™ Plus-Aerobic, BacT/Alert® SA, and BacT/Alert® FA bottles. However, when Gram-negative bacteria were extracted from BD BACTEC™ Plus-Aerobic blood culture bottles, the identification was also high for 0.5 µl of bacteria covered with DHB alone. The identification of Gram-positive cocci was moderate from BD BACTEC™ Plus-Aerobic bottles and negligible from BacT/Alert® SA and BacT/Alert® FA bottles. Pellets (0.5 µl) covered with DHB alone gave the highest identification rates, so that the application of a 0.5-µl pellet in combination with DHB treatment alone would be a suitable method for routine application for all bacteria.

Regardless of the type of blood culture bottle and protocol tested, direct identification by MALDI-TOF of Gram-positive bacteria was lower than for Gram-negative bacteria, being 60.0% versus 86.6%, 28.8% versus 69.2%, and 5.4% versus 47.1% for BD BACTEC™ Plus-Aerobic, BacT/Alert® SA, and BacT/Alert® FA, respectively. Identification from BD BACTEC™ Plus-Aerobic bottles yielded a difference of 26.6%. The difference for the identification of Gram-negative and Gram-positive bacteria of BacT/Alert® bottles was 40.4% for SA and 41.7% for FA. Although the result confirms recently published studies concerning the direct identification of Gram-positive isolates from culture bottles [2–10], which show differences ranging from 2% to 52%, the reason for the lower identification rate of Gram-positive bacteria has not been elucidated so far. As for MALDI-TOF MS analysis of bacteria grown on agar plates, this difference has not been described (the identification rate to the species level without protein extraction of Gram-negative aerobic bacteria is 70% and for Gram-positive aerobic cocci, it is 74%) [1]. In addition, of the Gram-positive cocci tested, the performance of the MALDI-TOF MS was better for the staphylococci than for the streptococci. And it was also better for the *S. aureus* and *S. epidermidis* than for the other coagulase-negative staphylococci. It is possible that the broth is an important environmental parameter that lowers the applicability of MALDI-TOF MS in the blood culture analysis of Gram-positive cocci in general and certain species in particular.

In summary, the results of the study presented here suggest that direct identification by MALDI-TOF MS from BD BACTEC™ blood culture bottles represents an efficient and rapid method for direct bacterial identification. However, direct identification from BD BACTEC™ blood culture bottles, especially for Gram-positive bacteria, has to be improved significantly.

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**Authors and contributors** Vera Schmidt designed and performed the experiments, analyzed the data, and prepared the tables; Anja Jarosch assisted in the MALDI-TOF MS analyses; Polina März and Charline Sander assisted in the performance of the experiments; Vladimir Vácata performed the statistical analyses; and Wiltrud Kalka-Moll designed the experiments, analyzed the data, and wrote the manuscript.

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