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Analysis and behavior of colistin during anaerobic fermentation

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HIGHLIGHTS

- A new LC–MS/MS method for colistin A and B in fermenter samples was developed.
- Anaerobic digestion of manure leads to a nearly complete elimination of colistin.
- An increase in methane production was observed in fermenters spiked with colistin.
- Anaerobic digestion is an efficient way to reduce environmental loads of colistin.

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ABSTRACT

A new analytical method for the determination of colistin in fermenter samples was developed followed by a study on the behavior of this substance during anaerobic fermentation. Analysis of colistin A and B was carried out by liquid chromatography–tandem mass spectrometry. Separation of the analytes was performed on a Security Guard column (4×3 mm). Fourteen fermentation tests in batch as well as in continuous reactors were carried out. After 44 days of anaerobic digestion of cattle manure, initially spiked with 500 mg/kg of colistin sulfate, a considerable decrease of the colistin concentration to less than 1 mg/kg could be observed. Furthermore, the daily production of biogas and methane was measured. A correlation between gas production and colistin concentration could not be determined. However, an increase of 10% of the cumulative methane production was observed in those fermenters spiked with an initial bolus of 500 mg/kg colistin.

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1. Introduction

As a consequence of intensive livestock farming the application of veterinary drugs has increased notably. Most of these substances are excreted unmetabolized and can be detected in manure (Martinez-Carballo et al., 2007). Subsequently, this manure is spread out as fertilizer on fields and grassland where the pharmaceuticals can adsorb to soil particles or leach into groundwater resources. Whereas no studies have been reported on the fate and behavior of colistin in the environment the persistence and accumulation of tetracyclines in soil was shown (Hamscher et al., 2002). Residues of these antibiotics in the environment bear the risk of building up antibiotic resistance in soil bacteria due to the stability and the detected concentrations of the active substances.

Colistin (also known as polymyxin E) is a polypeptide antibiotic, which belongs to the group of polymyxins. It is a complex mixture of at least 30 structurally similar polypeptides, of which 13 have been identified (Elverdam et al., 1981; Govaerts et al., 2003; Orwa et al., 2001). The basic chemical structure of all known colistin components is a cyclic heptapeptide with a side chain of three amino acids acylated at the N-terminus by a fatty acid. Colistin A (polymyxin E1) and colistin B (polymyxin E2) are the major components. Their structure was elucidated in the 1960s (Suzuki and Fujikawa, 1964; Suzuki et al., 1963a,b). Colistin A and B differ only in the fatty acid side chain.

In veterinary medicine colistin is applied orally via feed or drinking water and topically. The antibiotic mainly serves the treatment of diseases caused by gram-negative bacteria such as *Escherichia coli* or *Salmonella* (Falagas and Kasiakou, 2005).

Abbreviations: SPE, solid phase extraction; LC–MS/MS, liquid chromatography–tandem mass spectrometry; ESI, electrospray ionization; MRM, multiple reaction monitoring; HPLC, high-performance liquid chromatography; ODM, organic dry matter.

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Various analytical methods for the determination of colistin in biological matrices have been described. These include thin-layer chromatography (Thomas et al., 1980), microbiological (Leroy et al., 1989) and immunological methods (Kitagawa et al., 1985; Suhren and Knappstein, 2005), and capillary electrophoresis (Kristensen and Hansen, 1993). HPLC with either diode array or fluorescence detection is the most commonly used method for the analysis of colistin (Cancho-Grande et al., 2001; Decolin et al., 1997; Gmur et al., 2003). In recent years, tandem mass spectrometry (LC–MS/MS) has become the method of choice for a sensitive and selective detection of colistin and many other veterinary drugs.

Sample preparation is usually carried out by solid phase extraction (SPE) employing columns with modified silica (Decolin et al., 1997; Li et al., 2001) or polymers (Ma et al., 2008; Sin et al., 2005; Wan et al., 2006) as sorbent. Suhren and Knappstein (2005) suggested strong cation exchange sorbents, such as aromatic sulfonic acid. Only a few methods for the determination of colistin in food matrices have been published (Sin et al., 2005; Suhren and Knappstein, 2005). To our best knowledge, publications on the analysis of colistin in fermentation samples are not available. Mohring et al. (2009) developed a method for the determination of seven sulfonamides and trimethoprim in fermenter samples. Measurement of the antibiotics was carried out by HPLC–MS/MS after liquid–liquid extraction.

Today, manure is frequently used in biogas plants in the production of methane. Pharmaceuticals brought into the fermenter via the manure may have an influence on the fermentation process. On the other hand, the digestion process may have an effect on the antibiotics (Arikan et al., 2006; Mitchell et al., 2013; Mohring et al., 2009). Arikan et al. (2006) studied the effects of oxytetracycline during anaerobic digestion by using manure from calves with an initial antibiotic concentration of 9.7 mg/L. A reduction of the cumulative biogas yield of 27% was found. However, the percentage methane content of the biogas was unaffected. Sanz et al. (1996) reported a significant inhibition of the methane yield up to 80% during the fermentation of artificially prepared volatile fatty acids spiked with different levels of chlortetracycline (5, 40 and 152 mg/L) and chloramphenicol (25 and 50 mg/L). Recent studies on the influence of chlortetracycline at a concentration level of 28 mg/L manure showed similar effects (Stone et al., 2009). Decreases in biogas and methane yield caused by swine manure containing enrofloxacin in a concentration range from 16.7 to 1666.7 mg/L have also been demonstrated. In contrast, a significant increase in biogas and methane production could be obtained by spiking manure with 1 g of colistin/L (Zerr et al., 2012).

Furthermore, studies have shown that during anaerobic fermentation an elimination of antibiotics occurs. Arikan et al. (2006) determined a half-life of 56 days for oxytetracycline. Mohring et al. (2009) reported that some sulfonamides (sulfadiazine, sulfamerazine, sulfamethoxazole and sulfadimethoxine) and trimethoprim are eliminated by anaerobic digestion. In addition, a 4-OH-metabolite of sulfadiazine with reduced antimicrobial activity was identified in this study. Finally, Mitchell et al. (2013) exhibited a total removal of amphicillin, florfenicol and tylosin after less than 10 days of anaerobic digestion of cattle manure.

This is the first study to investigate the behavior of colistin during anaerobic fermentation. Therefore, a new sample preparation method for the quantification of colistin in fermenter substrate with HPLC–MS/MS was developed.

2. Methods

2.1. Chemicals and materials

Colistin sulfate as analytical standard and polymyxin B sulfate as internal standard were purchased from Sigma–Aldrich (Steinheim, Germany). The technical colistin sulfate standard was Belacol 100% Kompaktat and purchased from Bela-Pharm (Vechta, Germany). Methanol and acetonitrile (Merck, Darmstadt, Germany) were of analytical and HPLC grade. Formic acid, titriplex III (EDTA), citric acid monohydrate, di-sodium hydrogen phosphate and sodium hydroxide were of analytical grade and obtained from Merck (Merck, Darmstadt, Germany). All aqueous solutions and buffers were prepared by using ultrapure water from a Barnstead Easypure system (Thermo Fisher Scientific, Dreieich, Germany). Oasis HLB SPE cartridges (3 mL, 60 mg) were obtained from Waters (Eschborn, Germany) and plastic centrifugation tubes from Sarstedt (Nümbrecht, Germany). Samples were shaked on an LS 20 shaker (Gerhardt, Königswinter, Germany) and centrifuged in a Rotina 420 R tabletop centrifuge (Hettich, Tuttlingen, Germany). For evaporation of the solvent a vapotherm basis mobil II (Barkey, Leopoldshöhe, Germany) was used. The extraction buffer, 0.04 M EDTA (pH 4), was prepared by dissolving 10 g of citric acid monohydrate, 7 g of di-sodium hydrogen phosphate and 15 g of titriplex III in 1000 mL of ultrapure water and adjusting the pH with 5 M sodium hydroxide solution. The solution was stored at 4 °C.

2.2. HPLC–MS

The HPLC system (Agilent 1100 Series, Waldbronn, Germany) consisted of a quaternary pump, a degasser, an autosampler and a column oven set at 30 °C. Chromatographic separation was performed on a C18 Security Guard-guard column (4×3 mm) (Phenomenex, Aschaffenburg, Germany) at a flow rate of 0.42 mL/min under a gradient elution comprising of two mobile phases: 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Solvent A was held at 95% A for one minute, then changed to 5% A within 10 min and held for 10 min. At the end of each run the column was re-equilibrated with 95% A for 4 min before the next injection. The injection volume was 10 μL for all measurements.

A Quattro Premier XE tandem quadrupole mass spectrometer (Micromass-Waters, Eschborn, Germany) with an ESI source operated at positive mode was used for identification and detection of colistin A and B as well as polymyxin B1. For determination of the dominant precursor ions standard solutions (5 mg/L) of colistin and polymyxin B were infused separately via a syringe pump into the mass spectrometer at 10 μL/min. MRM was performed to determine product ion mass fragments of the identified precursor ions.

MS-MS analyses of colistin A and B as well as polymyxin B1 were carried out with the doubly charged ions. Tests performed during method development showed an approximately 35-fold higher signal intensity for colistin A and 30-fold higher signal intensity for colistin B compared to the single charged ions. For this reason the precursor ions were (m/z) 586 (colistin A), 579 (colistin B), and 603 (polymyxin B1) (Table 1), representing the molecular masses of 1170 g/mol for colistin A, 1155 g/mol for colistin B, and 1204 g/mol for polymyxin B1. Depending on the intensities of the precursor ions the tuning parameters were optimized. The desolvation temperature was set at 350 °C, dwell-time at 0.02 s and optimal capillary voltage was 3.05 kV. Argon (5.0) was used as collision gas and nitrogen as nebulizer gas. Nitrogen was provided by an in-house nitrogen generator with a purity of at least 99%. Substance specific parameters are given in Table 1.

2.3. Sample preparation and quantification

Frozen samples (2.4.1) were thawed in a water bath at room temperature within one hour. This was followed by a centrifugation step of 30 min at 3291g and 4 °C to remove solid sample components. 0.5 g of the liquid supernatant was used for sample preparation. Briefly, the supernatant was incubated with 9 mL of

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