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Sensing the impact of diet composition on protein fermentation by direct electrochemical NH_4^+ sensing in gastrointestinal digesta

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ABSTRACT

The correlation between nutritional habits and gut health directly impacts the gut-brain axis via a complex and not yet fully disclosed communication network. Establishing a link between our food intake and specific physiological responses as well as a better knowledge of diseases and the gut microbiota involves solving a challenging puzzle of biochemical pathways. Our understanding is limited by the inaccessibility of the gastrointestinal region to routine non-invasive chemical analysis. Here, we move a step further toward the direct assessment of a protein fermentation product, i.e., ammonium ions, via Ion Selective Electrodes (ISEs) in gastrointestinal digesta samples. By modulating the digestible protein content of the diet regimes of two groups of pigs, we discriminate the level of protein fermentation with a straightforward *quasi-in vivo* detection method which does not require any sample preparation. Our results show more than a 2-fold increase in ammonium ion concentration (from 180 ppm to 400 ppm) in the proximal colon for a diet based on poorly digestible proteins compared with a diet based on easily digestible proteins. Our approach shows good correlation with a standard laboratory technique for the determination of NH_4^+, i.e., the Indophenol Blue Method. Our results show the direct sensing of a protein fermentation product in a real matrix and demonstrate the great potential of potentiometric sensors to assess ammonium concentration profiles along the gastrointestinal tract for diets varying in protein digestibility and fermentation.

1. Introduction

Ion assessment in complex matrices represents a major focus in sensing technology with applications in different domains such as smart agriculture, water quality control, and medical screening (De Marco et al., 2007). In these applications, the fast and direct determination of analytes can have an important economic impact or enable the discrimination of severe clinical conditions even at early stage. Cations like sodium, potassium, calcium, lithium are fundamental in human physiological pathways and serve as indicators in many diseases (Felsenfeld et al., 2015). Another important physiological biomarker is ammonia (NH_3) which is mainly produced in the gastrointestinal (GI) tract with the colon accounting for most of its production (Barret, 2014; Ricci and Gregory, 2021). Here, during the fermentation of proteins by intestinal bacteria, ammonia is produced among other metabolites. In this physiological environment, ammonia is readily converted to ammonium ion (NH_4^+) according to a pH dependent equilibrium (Adlimoghaddam et al., 2016). It is widely accepted that the levels of NH_4^+ in the colon are associated with (undigestible) protein intake, establishing a clear link between dietary behavior and specific physiological mechanisms in the GI tract (Cummings and Macfarlane, 1991; Heo et al., 2010). The 'Western diet', which is characterized by low dietary fibers and protein-rich food, is generally considered to have detrimental effects on gut homeostasis and to trigger a pro-inflammatory environment (Malesza et al., 2021). However, biological mechanisms supporting these associations remain largely unknown and the *in vivo* exploration of the GI tract remains a challenge (Van Hellepute et al., 2020).

In this paper we explore the link between the dietary protein intake

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and the production of NH_4^+ by using an ion selective electrode directly in animal gastrointestinal digesta. Within the context of an animal nutrition study conducted by Wageningen University & Research, two groups of pigs (11 pigs in total) received a controlled amount of protein differing in digestibility with the aim to trigger protein fermentation of different extent, especially in the large intestine. The samples were then analyzed by means of a portable ISE setup. ISEs are potentiometric sensors converting the activity of an ion in solution to an electrical potential via an ion selective membrane. Due to their ease of operation and fast response time, they have become a very popular method in clinical chemistry, where the prime example is the pH electrode (Hemmink et al., 2010). Nevertheless, the application of ISEs for the detection of common ions like Na^+ or K^+ is not yet accepted as self-care diagnostic tool and it is solely designed for professional use (Dimeski et al., 2010; Hutter et al., 2022). However, if correctly operated, ISE can be a fast and reliable platform for point-of-care testing and even as real-time monitoring tool (Liu et al., 2022). In this study, we operate ISEs directly in animal digesta to validate this method in a real use-case with the main challenge being the harshness of this type of matrix. Our simple procedure does not involve any sample treatment and allows the direct determination of NH_4^+ on-site in *quasi–in vivo* conditions. The two dietary regimes are designed to trigger the production of different NH_4^+ levels and by correcting the NH_4^+-SE response for potassium ion interference using a secondary K^+-SE, we sense clearly distinct NH_4^+ levels for the two diets which was confirmed with standard laboratory analysis. This study demonstrates the great potential of potentiometric sensors as an analytical method in healthcare and nutrition making a step forward towards applying ISEs in complex matrices as handheld device.

2. Materials and methods

2.1. Animal ethics, diets, and digesta collection

The animal experiment was conducted at the research facilities of Wageningen University & Research (Wageningen, The Netherlands). This experiment was in the scope of a study aiming to evaluate nutritional quality of various protein sources for humans. All experimental procedures were approved by the Animal Care and Use Committee (DEC) of Wageningen University & Research and the Dutch Central Committee of Animal Experiments (AVD104002015326). Eleven pigs (Body Weight = 83 ± 7 kg) were individually housed in metabolic cages with *ad libitum* access to water (Hodgkinson et al., 2020). The pigs were divided into two groups, receiving different diets. To create a contrast in the protein fermentation level in the large intestine, diets contained similar levels of either poorly digestible collagen and zein (Low Digestible Protein diet, LDP) or easily digestible whey protein isolate (High digestible Protein diet, HDP), as reported in Table S1 (Calvez et al., 2021). The experiment consisted of a two-weeks adaptation period where pigs became acclimated to the diet. From at least 48 h before dissection, pigs were fed every 3 h and every 1 h for the last 6 h prior to dissection, to approach steady-state conditions in the gastrointestinal tract (David et al., 2014). One hour after the last meal pigs were sedated and exsanguinated as described in detail elsewhere (Martens et al., 2019). Afterwards, pigs were euthanized, dissected, and GI fluids were collected from the stomach (divided in proximal, stomach I, and distal, stomach II), (Bornhorst et al., 2013) small intestine (SI I & SI II: first and second half from beginning of small intestine to SI III, respectively, SI III: 1.5 m from ileo-cecal valve), cecum, colon divided by three equal parts as proximal, middle, and distal colon, and rectum. The contents of each segment were collected on ice by gentle finger stripping, homogenized, and subsampled. From each GI section, 20 mL of digesta was used directly for ISE measurements and 10 mL was immediately frozen at −20 °C after collection until laboratory analysis via the indophenol blue (IPB) method. Due to the low amount of digesta collected from SI III and rectum, these sections have been excluded from our investigation.

2.2. Chemicals and materials

Ammonium chloride (NH_4Cl), potassium chloride (KCl), sodium chloride (NaCl), sodium phosphate dibasic (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4), trichloroacetic acid ($C_2HCl_3O_2$), phenol (C_6H_6O) and sodium nitroprusside dihydrate ($C_5FeN_6Na_2O{\cdot}2H_2O$) were purchased from Sigma Aldrich and used without further purification.

2.3. Ion selective electrode (ISE) method

ISE were purchased from Sentek (Ammonium 3051 combination Ion Selective electrode and Potassium 3031 Combination Ion Selective electrode) and employed according to manufacturer recommendations. The specifications declared by the manufacturer are listed in Table 1. ISE measurements of GI samples were performed after collection. Since the response of potentiometric sensors depends on temperature, it is of utmost important that the calibration and measuring session take place at the same temperature thus, samples were allowed to reach room temperature in closed vials prior the analysis to prevent contamination. ISE measurements were performed directly on the raw samples without any pretreatment and repeated in triplicate. The commercial ISEs were employed with a Jenco 6250 handheld mV meter. Calibration solutions were prepared with a background of 100 mM sodium phosphate buffer (2300 ppm of Na^+) by mixing in the proper ratio the dibasic and monobasic phosphate salts in demineralized water (pH 6). Calibration standards of NH_4^+ (9, 36, 180 and 1800 ppm) were prepared from a stock solution of NH_4Cl in sodium phosphate buffer. The preparation of KCl standard solutions (39, 196, 782 and 1955 ppm) and NaCl solutions (230, 1150, 2300, 3450 ppm) followed the same protocol. The calibration step has been performed prior to the GI fluid measurements. Selectivity coefficients were determined with the Mixed Solution Method (MSM) (Umezawa et al., 1974) by measuring calibration curves at increasing interferent concentrations as displayed in Fig. S1. This pre-evaluation of the ISE performance has served to evaluate the selectivity coefficient of the NH_4^+-SE towards K^+ and it has been conducted under laboratory conditions. The pH of the GI digesta was determined by a commercial glass electrode from HORIBA Japan (9651S-10D).

2.4. Indophenol blue (IPB) method

The GI samples were analyzed for the ammonia content using the IPB method (Huizenga et al., 1994). In short, 5 g of each GI digesta was weighed and placed in a 50 mL falcon tube and mixed with 5 mL of trichloroacetic acid (TCA) 10% *w/v*. The resulting solution was mixed and centrifuged for 10 min and 2500 rcf. 1 mL of the supernatant was diluted with 1 mL of TCA 10% *w/v*. A 0.1 mL of the solution was then further mixed with 1 mL of phenolic solution prepared by mixing phenol with sodium nitroprusside in distilled water. The final concentration of the two reagents is 0.1 M and 0.2 mM for phenol and sodium nitroprusside, respectively. The resulting mixture was then treated by adding 1 mL of hypochlorite buffer solution and stored at 37 °C for 30 min in a water bath. The solution was removed from the water bath and cooled down till room temperature. Finally, each solution was analyzed with a

Table 1
Specifications of the combination Ion Selective Electrodes employed in this study (https://www.sentek.co.uk/). The table shows only the three main interferents for each ISE type.

ISE type	Concentration range (ppm)	T range (°C)	Main interference (Selectivity coefficient, K_{ij})	pH range
NH_4^+	0.9–9000	5–50	K^* (0.1), Na^* (2×10^{-3}), Mg^{2+} (2×10^{-4})	0–8.5
K^+	0.04–39000	5–50	Rb^* (2), Cs^* (0.4), NH_4^+ (0.01)	N/A

spectrophotometer (Evolution 201 from ThermoScientific) considering the absorbance variation of the peak at 623 nm.

2.5. Statistical analysis

Data collected with the ISE method in GI fluid are expressed as raw mean and standard deviation of the mean (SD). The linear correlation between the ISE and IPB methods was evaluated as R-squared. Data analysis was performed with MATLAB (calibration data) and OriginLab (statistical analysis).

3. Results and discussion

3.1. Characterization of the ion selective electrodes

Despite their versatility, ISEs are not free of drawbacks. They suffer from signal drift requiring frequent calibrations or, alternatively, the construction of a predictive model for drift compensation (Sisodia et al., 2022). In potentiometry, this is a routine step, but tedious if the sensor drift is unstable or accentuated. The selectivity of the ISE is ensured by the presence of an ionophore in the polymeric membrane having high affinity for the primary ion, i.e., the target analyte. However, the ISE response is affected by interference of other ions (Bakker and Pretsch, 2002; Lindner and Pendley, 2013). The selectivity coefficient K_{ij} defines the severity of the interference in the ISE response and it is determined experimentally. As reported in Table 1, K^+ and Na^+ are the main interferents of the NH_4^+-SE, and correction is recommended (Capella et al., 2020; Wang et al., 2020). Moreover, these two cations are abundant in the GI tract with K^+ values ranging from 250 ppm to 1300 ppm and Na^+ from 1000 ppm to 3500 ppm. Therefore, this effect must be taken into account, especially at low levels of NH_4^+ which are typical of the stomach and small intestine (Summerskill et al., 1966). The effect of K^+ and Na^+ interference on the NH_4^+-SE was determined by the MSM by measuring a series of NH_4^+ calibration curves at increasing concentration of interferents. As depicted in Fig. 1, the effect of K^+ on the NH_4^+-SE calibration curve is evident at low NH_4^+ concentration (9–50 ppm) where the linear profile bends upwards for K^+ concentration around 195 ppm.

This behavior is expected, and our MSM results confirm a K_{ij} of 0.14 (see Table 1). We determined negligible Na^+ interference and did not consider it further in this analysis (see Fig. S2). While the proper determination of NH_4^+ in GI digesta requires the concomitant measurements of K^+, K^+-SEs are insensitive to NH_4^+ at levels present in the GI tract (see Fig. S3).

3.2. Determination of NH_4^+ and K^+ in animal GI fluids

While indirect ISE sensing involves sample dilution and it is typically used in diagnostic laboratories, (Lewenstam et al., 1991; Megahed et al., 2019) here we evaluate fast, direct assessment of undiluted samples. The values of NH_4^+ recorded for each type of GI fluids are reported in Fig. 2 for the two groups of animals i.e., LDP diet group (Fig. 2a) and HDP diet group (Fig. 2b).

It is immediately evident that stomach and small intestine show lower levels of NH_4^+ compared to the large intestine, which is reasonable as NH_4^+ is a byproduct of protein fermentation which is taking place predominantly in the large intestine. The correction of the NH_4^+ values for K^+-interference is displayed in Fig. 2 with the grey columns. This correction has been performed for each subject on each GI section thanks to the concomitant determination of K^+ with a K^+-SEs (see Table S2 and Fig. S4). Considering the calibration curve shown in Fig. 1b, the correction of NH_4^+ concentration is necessary where low levels of NH_4^+ are present as observed in the initial sections of the GI tract. On the contrary, the correction is minimal where the concentration of the two cations is comparable like in the cecum section.

We compared the data obtained by the ISE method using the IPB method—the gold standard for accurate ammonia determination. IPB is based on spectrophotometric ammonia determination via the Berthelot reagent in basic conditions (Berthelot, 1859; Gordon et al., 1978; Zhu et al., 2019). The IPB method converts all available NH_4^+ into NH_3 by raising the pH of the sample. On contrary, the ISE method detects only NH_4^+ dissolved in the digesta, which represent the dominant species (>98%) since the pH of the samples lies always below 8 (see Fig. S5). Fig. 3 shows the direct correlation of the two analytical techniques in measuring the NH_4^+ levels in the different GI sections for the two diet groups.

The comparison of the two methods for the LDP diet shows a R-squared of 0.86 (Fig. 3a), while this value drops to 0.77 in the case of the HDP diet group (Fig. 3b). This good correlation also benefits from our K^+ interference correction. Without considering the matrix effect, the correlation of the two methods worsens (LDP, R-squared = 0.77; HDP, R-squared = 0.71; see Fig. S6). The lower R-squared associated with the HDP diet could be explained by considering the lower levels of NH_4^+ of the HDP group which in some cases approaches the lower limit of detection of our ISE and could result in higher uncertainty.

While the IPB method involves several sample preparation steps, our ISE approach relies on the fast and immediate assessment of the analyte with the scope to face the harsh environment and complexity of digesta samples and to bridge this technology to on-site analyte assessment. The close correlation of both methods demonstrates the applicability of ISE

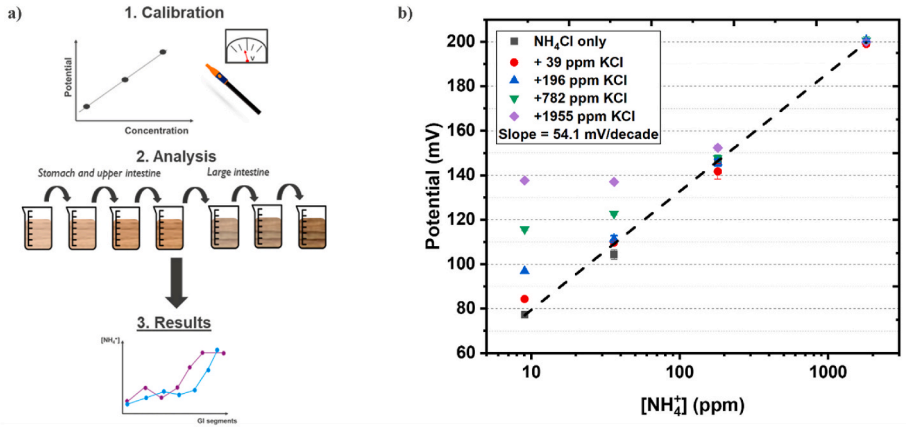

Fig. 1. (a) Sketch of the experimental approach. The ISE method has been applied directly to gastrointestinal digesta from pigs. (b) Calibration curve of the NH_4^+-SE showing the variation of the potential according to the concentration of NH_4^+ according to Nernst law. The colored traces show the behavior of the electrode in presence of increasing concentration of KCl according to the MSM. Error bars represent the standard deviation of triplicate measurements.

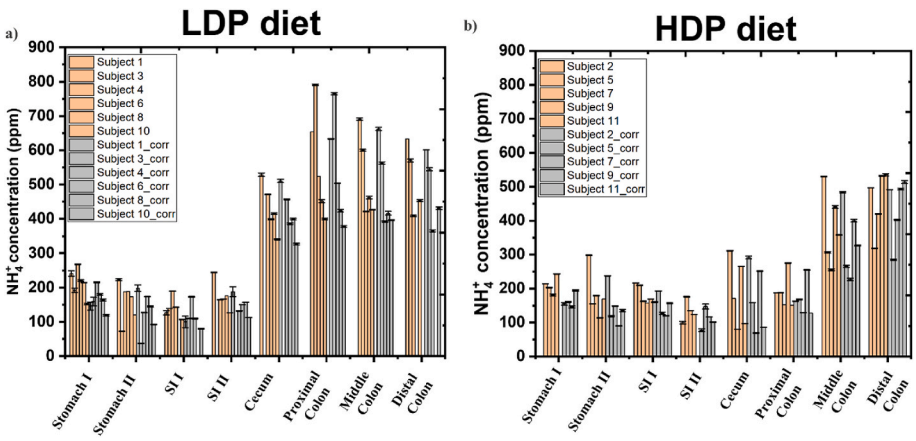

Fig. 2. NH_4^+ values recorded for each gastrointestinal section of each subject corrected according to the potassium levels. Orange columns indicate the raw NH_4^+ values, while the grey ones represent NH_4^+ values corrected for K^+ interference. Values of K^+ are reported in Table S2. (a) NH_4^+ values recorded from the subjects of the low digestible protein (LDP) diet group; (b) NH_4^+ values recorded from the subjects of the high digestible protein (HDP) diet group. Error bars indicate standard deviation resulting from triplicate measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

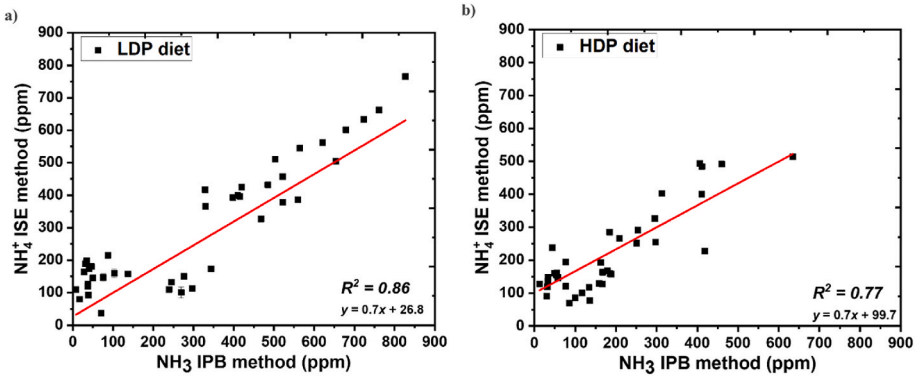

Fig. 3. Correlation between ISE and IPB method in the case of (a) LDP diet and (b) HDP diet. Black squares represent individual digesta samples from various gastrointestinal segments from 6 pigs in the case of the LDP diet and 5 pigs for the HDP diet. Error bars represent SD of the mean of triplicate measurements. The red line shows the linear correlation between the two sets of data. The stomach and small intestine sections of the gastrointestinal tract are located at the bottom left angle of the two graphs where the NH_4^+/NH_3 concentration is lower. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

as a valid technique for point of care testing (Lai et al., 2018; Megahed et al., 2019).

The lower correlation degree between the two methods of the HDP diet group is most likely related to the lower level of NH_4^+ associated with this diet. Despite this fact, the reproducibility of the ISE method is solid as confirmed by the low SD errors. In addition, our detection method combines the strong advantages of being an extremely intuitive approach and requiring no sample preparation.

3.3. *Effect of diet composition on NH_4^+ levels in the gastrointestinal tract*

In addition to validating the ISE method, the scope of this work poses fundamental questions about the role of NH_4^+ in protein fermentation. Intestinal microbiota is the primary responsible for protein fermentation with NH_4^+ as one of the main metabolites produced during the process. Production of ammonia in the large intestine stems from amino acid deamination or, to a lesser extent, urea hydrolysis by bacterial ureases (Blachier et al., 2007). Therefore the determination of NH_4^+ directly in GI fluid is crucial for establishing a correlation between the nutritional regime and the microbial activity of the large intestine. By using the LDP and HDP diet regimes, we aim at triggering different production of NH_4^+ at the cecal and colonic level (see Table S1 for diet compositions). The LDP diet contains poorly digestible zein and collagen (Hodgkinson et al., 2022) which result in a higher flow of undigested proteins into the large intestine which are broken down via proteolysis or fermented into amino acids by microbiota. This has shown to increase ammonia levels in the proximal colon in piglets (Pieper et al., 2012) and it is associated with higher levels of ammonia in human feces (Cummings et al., 1979; Geypens et al., 1997). In contrast, whey protein isolate is highly digestible resulting in a low amount of undigested protein entering the large intestine and thus in a low production of NH_4^+. With this premise, we expected a higher cecal and colonic production of NH_4^+ in the LDP group compared to the HDP group as a result of the low digestible protein content of the LDP diet (Calvez et al., 2021). Fig. 4 and Table S4 shows the NH_4^+ concentration profile of the two diets measured with ISE and IPB methods. As expected, in the stomach and small intestine microbial activity is lower, and the different diets did not trigger any differentiation in the levels of NH_4^+.

Moving to the cecum and proximal colon, we can clearly distinguish a difference between the two groups as expected. Particularly, the cecum is characterized by a higher concentration of NH_4^+ for the LDP (407 ± 54 ppm; $n = 6$) compared to the HDP group (185 ± 102 ppm; $n = 5$). This is also shown in the proximal colon where the LDP group results in a NH_4^+ concentration of 564 ± 159 ppm while the HDP group shows a NH_4^+ value of 191 ± 50 ppm. While the effect of the diet on the concentration of NH_4^+ is clearly visible, its effect on K^+ concentration is not observed

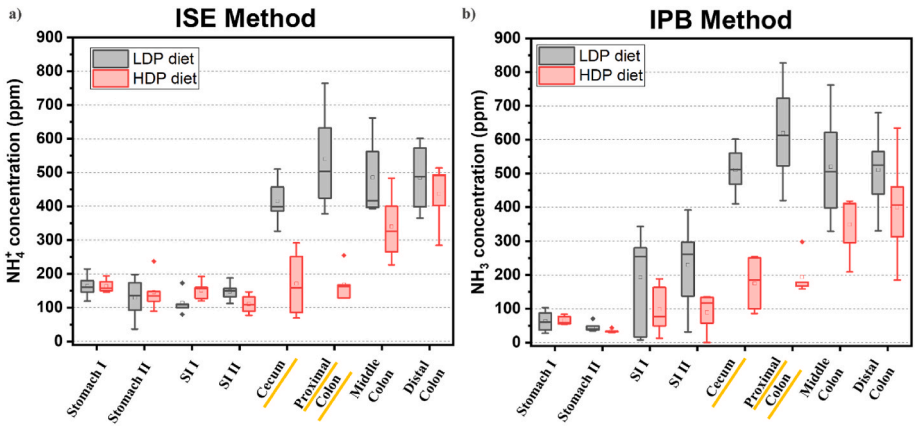

Fig. 4. NH_4^+concentrations profiles (in ppm) along the different GI tract sections, obtained with the ISE method and the IPB method. The two diets show significant difference in the NH_4^+ concentration in the cecum and proximal colon. Box charts indicate the minimum score, first (lower) quartile, median, third (upper) quartile, and maximum score. Mean and outliers are indicated by empty and filled spots, respectively. LDP n = 6, HDP n = 5.

(Table S3).

Another important observation is the overall trend of NH_4^+ among the different sections. The LDP diet group has a NH_4^+ concentration profile which shows an abrupt increase from the SI II to the cecum followed by a constant plateau while the HDP diet group shows a slower and progressive increase from cecum to distal colon. Notably, this trend has already been observed in humans (Macfarlane et al., 1992) and the reason why this phenomenon is not observed in the case of LDP group could be ascribed to a shift in microbiota preferences in the colon. The constant relative high flow of protein into the large intestine might have resulted in an increased abundance of proteolytic bacteria already at the level of proximal colon with consequent decrease of saccharolytic bacteria. Saccharolytic fermentation occurs primarily in the proximal colon, leaving the distal colon with low levels of fermentable carbohydrates. The low levels of fermentable carbohydrate in the distal colon also result in a higher pH, which makes the distal colon more favorable for proteolytic bacteria and to fermentation of undigested proteins (Korpela, 2018; Smith and MacFarlane, 1998). However, microbiota composition can change within days in response to dietary changes (David et al., 2014) and the pigs in the LDP group were fed with their diet for over 2 weeks before sampling.

If we now compare the ISE results from the two diet groups with the IPB method, the observed trend is confirmed, as displayed in Fig. 4b. The overall trend of NH_4^+ through all GI sections agrees with the results obtained with ISE method. As reported in Table S4, the correlation of the two methods considering the average NH_4^+ concentration for each gastrointestinal segment, results in an R-squared value of 0.86 in the case of the LDP diet ($n = 6$) and 0.82 for the HDP one ($n = 5$) (Table S4). The comparison confirms the capability of our method to evaluate NH_4^+ concentration profiles along the gastrointestinal tract for diets varying in protein digestibility and fermentation, even capturing the different trends of the large intestine sections. Finally, both the ISE and the IPB methods are capable of discriminating the two diets in the region of the cecum and proximal colon which are the only gastrointestinal sections showing a significant difference of NH_4^+ concentration. This result is unprecedented considering that gastrointestinal digesta analysis has previously been performed exclusively in laboratory conditions following a strict sample preparation protocol.

4. Conclusion

The gastrointestinal tract is ruled by a multitude of biochemical pathways which are directly affected by our nutritional habits. However, deep comprehension is hampered by the inaccessibility of the GI tract, and many questions about its function remain unanswered. Protein fermentation is an important process taking place in the large intestine and the catabolism of proteins by the microbiome results in NH_4^+ as one of the main by-products. By means of a dietary intervention, we have promoted different levels of NH_4^+ in the GI tract of two groups of pigs and performed a direct assessment of NH_4^+ by using ion selective electrodes. Our study has produced a consistent set of data that shows the NH_4^+ concentration profile in the different GI digesta, from stomach to distal colon. We demonstrated that ion-selective electrodes are well-suited for *direct* determination of NH_4^+ concentration in the very complex and harsh gastrointestinal tract—without any dilution or other sample preparation. Comparison with a classical spectrophotometric method results in a very good agreement between the two methodologies. In addition, we were able to define a characteristic NH_4^+ concentration profile for the two diet groups, namely LDP and HDP, finding significant differences between the diets in protein fermentation in the region of cecum and proximal colon. The quasi-*in vivo* assessment of NH_4^+ levels in GI digesta shows a clear discrimination of diets using the ISE method. These results demonstrate the power of potentiometric sensors to detect relevant biomarkers in undiluted GI samples, helping to unravel the link between nutrition and GI metabolic functions. While our method demonstrates a clear application in a real use case, the next step of this technology is the translation to *in vivo* conditions. This challenge requires substantial miniaturization and careful control of sensor fouling as well as reference electrode potential drift. Potentiometric sensors are suited well for miniaturization. Future work in overcoming fouling on the other hand requires a deep knowledge of the sensor response in the specific matrix of investigation as well as a correction for matrix effects and drift.

Credit author statement

Francesca Leonardi and Ria R. Sijabat: Methodology, Experiment and data collection, Writing – original draft. Roseanne Minderhoud: Experiment and data collection, manuscript optimization. Aniek J. G. Even: Conceptualization, Experiment and data collection. Klaus Mathwig: Data Analysis, Manuscript optimization. Rachel E. Armstrong: Experiment and data collection, manuscript optimization. Sonja de Vries: Conceptualization, Manuscript optimization. Annelies Goris: Conceptualization and manuscript optimization. Chris Van Hoof: Conceptualization and manuscript optimization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biosx.2023.100406.

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