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Anaerobic degradation of *N*-ε-Carboxymethyllysine, A Major Glycation End-Product, By Human Intestinal Bacteria

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Abstract

Modifications of lysine contribute to the amount of dietary advanced glycation end-products reaching the colon. However, little is known about the ability of intestinal bacteria to metabolize dietary *N*-ε-carboxymethyllysine (CML). Successive transfers of fecal microbiota in growth media containing CML were used to identify and isolate species able to metabolize CML under anaerobic conditions. From our study, only donors exposed to processed foods degraded CML and anaerobic bacteria enrichments from two of them used 77% and 100% of CML. *Oscillibacter* and *Cloacibacillus evryensis* increased in the two donors after the second transfer highlighting bacteria from these taxa could be candidates for anaerobic CML degradation. A tentative identification of CML metabolites produced by a pure culture of *Cloacibacillus evryensis* was performed by mass spectrometry: carboxymethylated biogenic amines and carboxylic acids were identified as CML degradation products. The study confirmed the ability of intestinal bacteria to metabolize CML under anoxic condition.

Keywords: Microbiota, Maillard reaction, *N*-ε-carboxymethyllysine, Intestinal metabolism, Dietary Advanced Glycation End-products,

Introduction

Thermal treatment of foods is well known to result in the formation of Maillard reaction products, also named dietary advanced glycation end-products (d-AGEs). [1] Free amino groups of proteins and amino acids react with reducing carbohydrates, forming compounds that are poorly bioavailable and cannot be used for the *de novo* protein synthesis by the human body. In some foods, Maillard reaction (MR) is limited to the initial stages and the main detectable products are Amadori and Heyns compounds. [2] In severe thermally treated foods, reaction products such as hydroimidazolones, pyrraline, hydroxymethylfurfural and *N*-ε-carboxymethyllysine (CML), are predominantly found.[3] CML is a particularly stable compound that was found in the range 0.01-5.09 mg/100g in many different foods, as dairy products, bread crust, cookies.[4] Due to its chemical nature and ubiquity presence in foods and *in vivo,* CML has been largely used as a biomarker of the dietary intake thermally treated foods and it is probably the most investigated d-AGE.[4]

The nutritional and health concerns of thermally treated foods are related to the decrease of available lysine and arginine and to the metabolism of the d-AGEs. [5, 6] A substantial percentage of lysine can react with carbohydrate through MR and since most of the d-AGEs-containing proteins are not bioavailable, they become part of unabsorbed digesta that reaches the colon every day.[7] This is not only due the direct lysine blockage, but also a consequence of the fact that glycation and oxidation promote significant protein crosslinking decreasing the overall digestibility and funneling more material to the gut. [8] As a result, these d-AGEs serve as substrate for intestinal bacteria. The adoption of a diet with plenty of cooked and processed foods has surely be beneficial to increase the energy intake. [9] The ability of microbiota to use d-AGEs may provide an additional advantage to individuals harboring suitable species.

Nevertheless, animal studies have shown that some of the d-AGEs are potentially toxic and their degradation may contribute to the general health of the consumer. [10]

The ability of the intestinal microbiota to metabolize glycated proteins has become of general interest as part of the larger discussion on the metabolism of dietary protein by the colonic bacteria. [11] Mounting evidences suggest that a diet rich in glycated proteins profoundly changes the intestinal microbiota composition. Recently, it was reported that one of the intermediates of the MR, *N*-ε-fructosyllysine, could no longer be detected after 4 h of incubation in fecal material from healthy volunteers who consumed a Western diet. [12] *Intestinimonas butyriciproducens* was isolated from stool of a healthy individual and this strain is able to convert *N*-ε-fructosyllysine into butyrate highlighting microbiota responses to dietary component. [13, 14]

Our present insights in the capability of human microbiota to metabolize CML under anaerobic conditions are very limited. The first pivotal work estimated that 10% of the orally ingested AGEs was absorbed and only 30% of that was eliminated in the urine. [15] It was also observed that CML and *N*-ε-fructosyllysine are available for absorption during simulated gastrointestinal digestion, while lysinoalanine and other crosslink products are not. [16] Both d-AGEs appear as small peptides similar to proteinogenic amino acids and have similar biochemical characteristics. Interestingly, it was found that dietary CML (d-CML) is partially bioavailable and a significant amount is accumulated in various organs. [17] It is known that d-CML does not bind to the receptor of AGEs (RAGE) as is the case for the endogenously formed CML, which is formed by the lysine present in collagen and other tissue protein. Hence, the mechanisms responsible for the inflammatory action of d-CML are not known and no scientific consensus has been reached on the physiological relevance of CML so far. [18] Besides accumulation in the organ and urinary excretion, metabolism of d-CML showed that the large majority is recovered in the feces: the consumption of the d-AGEs-high diet led to a higher CML input associated

with a higher fecal excretion in a two periods crossover trial with 11–14 years adolescent males. [19] Recent data showed that d-CML is partially metabolized by the gut microbiota. [12] Notably, it has been shown that under aerobic and low amino acids conditions, in presence of CML dipeptide and CML, five different strains of *Escherichia coli* were able to linearly produce three CML bacterial metabolites up to 8.4% of initial CML dose, *N*-carboxymethylcadaverine (CM-CAD), *N*-carboxymethylaminopentanoic acid (CM-APA), and the *N*-carboxymethyl-Δ^1-piperideinium ion. [20] However, the involved anaerobic intestinal microbes have not been identified nor isolated or characterized.

In this work, we aimed at studying the capability of intestinal bacteria to degrade dietary CML. A strategy of successive transfers of fecal microbiota in growth media containing CML as major carbon source was adopted to enrich, identify and isolate the anaerobes able to metabolize d-AGEs and corroborate previous findings on CML degradation pathways.

Material and Methods

Chemicals and reagents

Acetonitrile, methanol and water for mass spectrometry analyses were obtained from Merck (Darmstadt, Germany). Analytical standards *N*-ε-carboxymethyllysine (CML), Nε-(carboxy[$^2H_4$]methyl)-L-lysine (d_4-CML) were obtained from TRC-Chemicals (Toronto, Canada), while ammonium hydroxide solution (28% in water w/v), ammonium acetate, formic acid, lysine and L-lysine-4,4,5,5-d4 hydrochloride (d_4-Lys) were purchased from Sigma (St. Louis, MO)

Enrichment and growth media

The study involved 6 subjects that donated fecal samples **(Table 1)**. Informed consent was obtained from the mothers for their approximately 6 month-old infants and the Asian adult, while the two African samples were derived from a previously reported intervention study and preserved in 25% glycerol (kind gift from Prof O'Keefe, Pittsburgh' USA). [21]

Fresh fecal samples were collected in 15 mL Falcon tubes containing anaerobic phosphate buffer (pH 7.0) and later stored in 25% glycerol in 5 mL anaerobic bottles kept at -80 ^{o}C. Aliquots (0.5 mL) of these fecal slurries were transferred to 10 mL anaerobic bicarbonate-buffered mineral salt medium (CP medium- see below) containing CML as energy and carbon source for the first enrichment. CML concentration at time 0 was reported in **Table 2**. These anaerobic bottles were filled with a headspace of CO_2/N_2 (1:4) at 1.5 atm and incubated at 37 ^{o}C. Subsequently, 0.5 mL of the fully grown first enrichment was transferred to 10 mL of fresh CP medium containing CML as in a 10 mL bottle with a head space and incubation regimen as before.

The growth experiments on the CML degradation capacity of the newly isolated *C.evryensis* strain AS3 was also performed in CP medium (see below) containing either 5 mM CML alone or 5 mM CML with a combination of 10 mM lysine, 10 mM serine, 10 mM histidine and 10 mM arginine. Lysine, serine, histidine and arginine were individually added to CP medium from 0.5 M sterile anoxic stock solutions upon inoculation, while CML was added to the medium as powder and filter sterilized afterwards. The cultures were sampled at regular intervals for CML analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Enrichments were performed in anaerobic bicarbonate-buffered mineral salt medium (CP medium) consisting of: 0.53 g/L $Na_2HPO_4.2H_2O$, 0.41 g/L KH_2PO_4, 0.3 g/L NH_4Cl, 0.11 g/L $CaCl_2.2H_2O$, 0.10 g/L $MgCl_2.6H_2O$, 0.3 g NaCl, 4.0 g/L $NaHCO_3$ and 0.48 g/L $Na_2S.9H_2O$ as well as alkaline and acid trace elements (each 0.1% v/v) and vitamins (0.2% v/v).[22] The alkaline trace element solution contained the following (mM): 0.1 Na_2SeO_3, 0.1 Na_2WO_4, 0.1 Na_2MoO_4 and 10 NaOH. The acid trace element solution was composed of the following (mM): 7.5 $FeCl_2$, 1 H_3BO_4, 0.5 $ZnCl_2$, 0.1 $CuCl_2$, 0.5 $MnCl_2$, 0.5 $CoCl_2$, 0.1 $NiCl_2$ and 50 HCl. The vitamin solution

had the following composition (g/L): 0.02 biotin, 0.2 niacin, 0.5 pyridoxine, 0.1 riboflavin, 0.2 thiamine, 0.1 cyanocobalamin, 0.1 *p*-aminobenzoic acid and 0.1 pantothenic acid.

Isolation of anaerobes was further performed by streaking on plates of Reinforced Clostridium Medium (BD) solidified with 1.5% agar that were incubated in anaerobic jars filled with CO_2/N_2 (1:4) at 1.5 atm. The picking and streaking were performed in an anaerobic tent filled with N_2/H_2 (95/5 v/v).

Volatile fatty acids detection

The formation of volatile fatty acids and alcohols was measured on a Thermo Scientific Spectra HPLC system equipped with P2000 pump; AS3000 autosampler an Agilent Metacarb 67H 300 × 6.5-mm column kept at 45 °C and running with H_2SO_4 as an eluent. The detector was a RI-150 refractive index detector. The eluent flow was 0.8 mL/min. The bacterial cultures were centrifuged at 14000g for 5 min at room temperature to obtain the supernatants of which 400 µL was used to analyze in the HPLC. The measurement was performed at 45 °C for separation of volatile fatty acids and alcohols. [23]

CML and free lysine detection by liquid chromatography tandem mass spectrometry

Free lysine and CML were analyzed in order to combine target analysis and CML degradation products investigation; 0.5-mL cultures of the enrichments were collected at different time intervals up to 5-6 weeks. Upon the sampling, the supernatants were collected by centrifugation at 21000g for 10min at 4 °C and subsequently diluted five hundred times in acetonitrile: water (50:50, v/v) and 5 µL was used for injection. Hydrophilic interaction liquid chromatography (HILIC) separation of CML, lysine d_4-lysine and d_4-CML was achieved on a thermostated (40 °C) Luna amino column (3.0 µm, 100 x 2.0 mm, Phenomenex, Torrance, CA) equipped with an amino security guard (4.0 x 2.0 mm). The following mobile phases were used: A, acetonitrile and B, 20 mM ammonium acetate in water with ammonium hydroxide added to adjust alkalinity

(pH 9.0) according to the procedure that was optimized previously. [24] The compounds were eluted at 300 µL/min through the following gradient of solvent B (t in [min]/[%B]): (0/2), (2.5/2), (7/90), (9/90). Positive electrospray ionization was used for detection and the source parameters were selected as follows: spray voltage 5.5 kV; capillary temperature: 400 °C, dwell time 100 ms. The chromatographic profile was recorded in multiple reaction monitoring mode (MRM) by using an API 3000 triple quadrupole (ABSciex, Carlsbad, CA). Target analytes and their labeled internal standards were analyzed using the mass transitions given in parentheses and in bold the transition used for the quantitation in the case of target analytes and for qualification in the case of internal standards experiments: lysine (*m/z* 147 → **84**, 130), d_4-lysine (*m/z* 151 → 88, **134**), CML (*m/z* 205 → **84**, 130), d_4-CML (*m/z* 209 → **88**, 134). Analytical performances robustness, sensitivity, reproducibility, repeatability, linearity, accuracy, carry over and matrix effects were evaluated by following the procedures previously reported. [25] Typical retention times were 4.5 min, and 5.3 min for lysine and CML respectively. Quantitation of CML and lysine was performed by using the internal standard technique, results were reported as mmol/L.

Identification of CML degradation metabolites

An internally generated database of CML degradation metabolites was constructed following lysine metabolite entries from the publicly available online metabolite databases KEGG (www.genome.jp/kegg/) and adding a carboxymethyl group (-CH_2COOH) to the lysine amino side chain. Tentative identification of CML degradation products was achieved by high-resolution mass spectrometry (HRMS). Optimal conditions for chromatographic separation of CML metabolites were obtained after two consecutive trials by using amino column with mobile phases as described above for tandem mass spectrometry quantitation of CML and by means of a silica sulfobetaine zwitterionic modified HILIC column (50 x 2.1 mm, 3.0 µm, Thermo Fisher,

Bremen, Germany) at 35 °C. Mobile phases for zwitterionic column were 0.1% formic acid in acetonitrile (solvent A) and 0.1% formic acid in water (solvent B). Samples were diluted ten times in a mixture acetonitrile: water 50:50 (v/v) and the following gradient of solvent B (minutes/%B): (0/10), (0.80/10), (3.5/5), (5.5/5) was used. The flow rate was set to 300 µL/min and the injection volume was 5 µL. The U-HPLC system (Accela 1250, Thermo Fisher, Bremen, Germany) was interfaced to an Exactive Orbitrap HRMS (Thermo Fisher, Bremen, Germany), equipped with an heated electrospray source (HESI-II) working in polarity switching mode. The current ion of potential candidates was scanned in the *m/z* range of 50–350 by using a scan time of 1s. Interface parameters were: spray voltage 4.2 kV, capillary voltage 35.0 V, capillary temperature 350 °C, heater temperature 300°C, sheath gas flow and auxiliary gas flow were 42 and 12 arbitrary units, while for negative acquisition mode: spray voltage -3.8 kV, capillary voltage -25.0 kV. HRMS conditions were optimized by infusing a mixture of CML, d_4-CML, lysine and d_4-lysine (20 µg/mL) at a flow rate of 3 µl/min. Mass tolerance was fixed at 5 ppm and the exact mass 83.06037 $[M_2+H]^+$ of acetonitrile was used as recalibrating agent (lock mass) to improve selectivity and signal stability. Analyte concentrations were monitored by external standard technique with CML as standard and the intensities, signal to noise ratio (S/N) stability and reproducibility among different batches of samples were used to select between amino and zwitterionic column. Metabolites concentration was reported as µmol/L

Bacterial community analysis

The cell biomass was harvested from an aliquot (1-2 mL) of well-homogenized liquid culture by centrifuging at 13,000g for 10 min. DNA was extracted from the pellet by using MasterPure™ Gram Positive DNA Purification Kit (Epicentre, Madison, United States) according to manufacturer's instructions. The PCR was performed using 27F and 1492R to amplify the complete 16S rRNA genes of the bacteria using the program starting at 94 °C for 5 min and

continued with 35 cycles consisting of 94 °C for 90 s, 52 °C for 30 s and 72 °C for 90 s and finally 72 °C for 10 min. [26] The PCR products were subsequently purified by PCR purification kit (Qiagen, Hilden, Germany) and used to generate a clone library of full-length 16S rRNA gene sequences using pGEM Easy Vector Systems (Promega, Madison, United States). All steps mentioned above were done following the manufacturers' instructions. Twenty-four to sixty clones were selected for sanger sequencing at GATC Biotech (Konstanz, Germany) using SP6 (5´-ATTTAGGTGACACTATAGAA-3´) as sequencing primer. The sequences were trimmed with DNASTAR to remove vector contamination and manually checked. Later they were aligned with the multiple sequence aligner SINA and merged with the Silva SSU Ref database (release 111). Phylogenetic trees were constructed in the ARB software package (v. 6) by the same algorithm. [27]

Results and Discussion

CML enrichments from stool

Fecal samples from 3 infant and 3 adult volunteers **(Table 1)** were enriched directly in bicarbonate-buffered medium containing CML as the only carbon and energy source without any intermediate step or substrate, under anaerobic conditions. The first enrichment showed that the fecal microbiota of all volunteers were able to degrade CML, however at very different levels **(Table 2)**. While the microbiota of adult donors AF1A and AS1 degraded this substrate the best after 5 weeks incubation, that of M2 and AF4A had the lowest CML degradation capabilities. The degradation was rather slow and this could be attributed to a non-optimized medium for growth of potential CML degraders. The microbiota of the infants (both breast and formula fed) showed very limited CML degradation capacity. Among the three adults, two showed a good degradation capacity and one did not. This is in line with the possibility that dietary exposure to CML is connected to the microbiota ability to metabolize it.

Interestingly, CML degradation activities of the samples from donors F1, AF4A, M1 and M2 were not detected in the second transfer while those of samples AF1A and AS1 were somewhat reduced (see below). This may indicate that either some growth factors were absent or microbial partners involved in CML degradation were missing at the second transfer.

To get further insights in the bacteria that were able to degrade CML, we focused on the incubations of the samples from donors AF1A and AS1 as these were two samples with the highest degradation activity (77% and 100% of the added CML, respectively). The first enrichments were transferred a second time and the bacterial composition in the two CML enrichments were determined using Sanger sequencing of clone libraries of 16S rRNA amplicons. Sequences of approximately 900 bp obtained were subsequently used to construct phylogenetic trees and assign taxonomic groups.

Microbial composition analysis indicates uncultured *Oscillibacter* spp as potential CML degrader.

The microbial composition of the first and second enrichments was determined to investigate the microbes that were involved in anaerobic CML degradation in the fecal sample of volunteer AF1A **(Figure 1)**. After 5 weeks incubation, 3.5 mM CML were degraded in the first enrichment while 1.2 mM CML was utilized in the second transfer. In the first enrichment, *Clostridium butyricum, Ruminococaceae* and *Oscillibacter* were the most dominant while after the second transfer the majority of the enrichment belonged to *Oscillibacter*, *Clostridium butyricum, Clostridium sphenoides* and *Escherichia coli* with *Oscillibacter* spp. was the most prevalent taxon **(Figure 1)**. Based on the clone library abundance, the sequence of *Oscillibacter* spp. made up 10.7% of the total microbial community in the first enrichment and increased to 35.6% after the second transfer. The typical morphology of *Oscillibacter* was also observed in the first and second enrichment (**Figure 1-C**).[28] Up to date, there is no report about anaerobic bacteria

that are able to degrade CML in anoxic condition. However, an increase in abundance of *Oscillibacter* spp.in the second transfer indicated a possible role of this bacterium in anaerobic CML degradation. So far, efforts to isolate this bacterium from this enrichment using different media have not been successful in line with the reported difficulties in culturing members of the genus. [29]

Complete degradation of CML by an enrichment of bacteria related to *Cloacibacillus evryensis*

The microbiota in the fecal sample from volunteer AS1 showed the capacity to completely degrade CML in the first enrichment **(Table 2)**. While more than 2 mM CML was completely degraded in 5 weeks in the first enrichment, only 0.8 mM CML was degraded in the second enrichment **(Figure 2B)**. As with the enrichment of the fecal samples of volunteer AF1A, this suggested that some growth factors present in the fecal sample could be missing in the second enrichment. Interestingly, the microbial community analysis of the second enrichment of the fecal sample of volunteer AS1 enrichment revealed an up to 80% enriched population of a single taxonomic group, *Cloacibacillus evryensis* **(Supplementary Figure 1) (Figure 2A)**. This species has been known as an active amino acid degrader in a mesophilic anaerobic digester. [30, 31] However, *C.evryensis* has not been isolated yet from a human specimen, although its presence has been suggested in stools of human and other animals. The CML degradation results in the second transfer of AS1 enrichment also indicated that bacteria related to *C.evryensis* could well be responsible for the anaerobic CML degradation.

***Cloacibacillus evryensis* isolate with CML degradation capacity**

We made several attempts to isolate the enriched bacteria related to *C.evryensis* by plating the enrichment on Reinforced Clostridium agar. After picking and streaking colonies several times, we got an axenic culture, termed *C.evryensis* strain AS3 since a 16S rRNA gene sequence of

the pure culture showed >99% similarity to that of the type strain of *C.evryensis* strain DSM 19522^T. [30] Moreover, this sequence was identical to that detected in the clone library **(Figure 2A)** indicating that *C.evryensis* strain AS3 was representative of the enriched cells. The cells of *C.evryensis* strain AS3 were short rods and often grown in duplococci **(Figure 3B)**. Interestingly *C.evryensis* strain AS3 was not able to grow in glucose but could grow on a mixture of amino acids including lysine, histidine, serine and arginine producing propionate and acetate as major end-products. As *C.evryensis* strain AS3 was able to ferment lysine, histidine, serine and arginine like other *Cloacibacillus spp*., a CML degradation test was performed in two conditions with and without these amino acids under anaerobic conditions **(Figure 3A)**. Without lysine, histidine, serine and arginine, *C. evryensis* strain AS3 was able to slowly degrade CML, but in the presence of amino acids mixture, CML degradation was somewhat faster, with acetate and propionate as main end-products under both conditions. This result indicated that the pure culture of *C. evryensis* AS3 was able to degrade CML anaerobically. The slow CML degradation might be explained by a number of factors, including lack of growth factors, potential syntrophic partners, or the formation of toxic CML degradation compounds that inhibit the growth/activity of the bacteria. Of note, we tested the growth of *C. evryensis* strain AS3 in several rich media but it never grew to a high OD of over 1 even in an optimized medium. [30] Moreover, a recent study on CML degradation showed that *E.coli* was able to degrade only a limited part of an initial low concentration (250μM) of CML at aerobic conditions, while producing 3 different metabolites. [20] In our study, much higher CML concentrations were used suggesting the possibility that *C. evryensis* strain AS3 may also degrade initial low concentrations of CML.

Identification of potential markers of CML degradation products

The formation of CML degradation products was investigated by zwitterionic HILIC and HMRS in bacterial cultures showing CML degradation capacity. Chromatographic conditions were

optimized by means of acetonitrile and water in acidic environment (0.1% formic acid) in order to improve ionization in positive ion mode as well as retention and separation of metabolites from their precursor CML. Several compounds were considered as potential candidates of CML degradation and included in the *in-house* database according to KEEG pathway. Full-scan analysis in high resolution polarity switching mode (*m/z* range 50-350, resolving power set to 50,000 full width at half maximum FWHM, *m/z* 200) was considered as the optimal strategy to combine the chemical nature of the potential candidates to their behavior in HILIC chromatography. [32] A total of four out of twenty-five candidates were tentatively identified as potential markers of CML metabolism and included *N*-carboxymethylcadaverine (CM-CAD), *N*-carboxymethylaminopentanoic acid (CM-APA), 2-amino-6-(formylmethylamino)hexanoic acid and *N*-carboxymethyl-Δ^1-piperideinium ion. The analytical characteristics of these indicator compounds were determined **(Table 3)**. The use of HILIC-HRMS allowed the separation of CML metabolites from their precursor, based on the typical retention time and partition coefficients: three out of four compounds exhibited a logP below 0 resulting in a capacity factor higher than 2 in the chromatographic conditions tested.

To test the usefulness of these four indicator compounds, we analyzed their presence during the degradation by *C. evryensis* strain AS3 of CML **(Figure 4)**. Data indicated an overall accumulation trend for the four metabolites during the time course of incubation with AS3 strains, in particular up to the first five weeks. The sum of the four metabolites explained only the 1.5% of the CML degradation suggesting that other metabolites not included in target HRMS analysis experiments could be formed, as well as a dose-dependent relationship between metabolites formed and initial concentration of CML. Besides the multitude of compounds arisen from lysine degradation mediated by *Cloacibacillus* metabolism, the presence of a carboxymethyl group on the epsilon side chain of lysine and its steric hindrance limited the

number of potential candidates included in our *in-house* database. The concentration of CM-CAD remained constant during incubation in presence of lysine, arginine, serine and histidine suggesting that long incubation may promote reaction of primary amino group leading to carboxymethylation of cadaverine arising from lysine metabolism. Vice versa, when the microbial culture enriched in *C. evryensis* strain AS3 was incubated in presence of CML, concentration of CM-CAD increased up to 86% during the first 17 days toward initial concentration. Similar trends were observed for CM-APA: in the case of CML incubation with other amino acids source, the formation of the metabolite was characterized by an increase up to 3.36 ± 0.26 μM after 30 days then it constantly decreased at the end of the incubation. In the case of incubation with CML alone, formation of CM-APA was characterized by a decrease after the first 30 days, suggesting that the presence of other amino acids source had a key role for the yield of CM-APA. Formation of *N*-carboxymethyl-Δ^1-piperideinium ion was substrate specific: only CML degradation led to its formation, while the degradation of other amino acids did not have an influence on the formation of this ion. Indeed *N*-carboxymethyl-Δ^1-piperideinium ion formation exhibited similar trend in presence of co-factor suggesting that its formation was linked only to the presence of CML as carbon source able to generate piperidein ions. The 2-amino-6-(formylmethylamino)hexanoic acid is the only metabolite having a completely different trend in presence of other amino acids respect to CML alone. When other amino acids are present its concentration sharply increased during the first month, while in presence of CML alone the concentration suddenly dropped during the first days. This figure suggests a potential interconversion in the catabolic pathways of different amino acids.

Lysine degradation pathway and anaerobic conditions were used as reference to tentatively explain CML metabolism. As depicted in **Figure 5**, decarboxylation of the alpha carboxylic group mediated by lysine decarboxylase is one of the possible routes that led to the formation

of CM-CAD. The formation of *N*-carboxymethyl-Δ^1-piperideinium ion could include CM-CAD as precursors: in this respect a reductive deamination followed by an intramolecular cyclization of the epsilon amino group was the key step for piperidein derived compound formation. On the opposite, the formation of CM-APA, required a preliminary amide formation followed by the oxidation into the corresponding carboxylic acid. This mechanism can be catalyzed by 5-aminopentanamidase similarly to lysine metabolism. The formation of 2-amino-6-(formylmethylamino)hexanoic acid requires the presence of a carboxylic acid oxidase for the formation of the aldehyde. Once aldehyde is formed, there is a possibility of a reduction of the aldehyde into the corresponding alcohol through the Ehrlich pathway. [33]

Post-translational modifications of amino acids and the consequent formation of Amadori compounds, Heyns compounds and d-AGEs contribute to the variety of compounds metabolized by the intestinal microbiota. [34] The identification of markers of d-AGEs metabolism is as intricate as the Maillard cascade and multiple factors need to be considered before depicting the appropriate metabolic pathway. Even if aerobic conditions favored a faster CML degradation by *E.coli* strains, anaerobic environment is not a limitation and similar compounds can be obtained as end-products (or intermediate) during incubation with fecal microbiota. For the four compounds identified, the presence of other amino acids along with CML was not determinant and no significant difference in the yields were obtained except 2-amino- 6-(formylmethylamino) hexanoic acid and CM-APA after 5 weeks. In agreement with previous results, the appropriate substrate can be relevant to determine the optimal conditions for CML metabolism; [20] in the present paper we demonstrated that successive transfer of fecal microbiota are able to generate CML metabolites following similar pathways of lysine degradation. As lysine is a preferred substrate of colonic bacteria and different pathways are responsible of its catabolism, when hypothesizing parallel pathways between lysine and CML,

multiple conditions needs to be taken into account, as time of incubation, pH changes and presence of co-factors. [35] Finally, our results in anaerobic conditions are in line with aerobic incubation of CML in presence of *E.coli* strains, the concentration of CM-CAD was close to 10 µM (around 4% of 250 µM of CML) detected by Hellwig and coworkers, while CM-APA was one order of magnitude higher meaning that its formation prevailed in anaerobic conditions, while a key role can be attributed to *N*-carboxymethyl-Δ^1-piperideinium ion in anaerobic condition as its concentrations throughout the incubation were three and four times higher than CM-APA and CM-CAD, respectively. [20]

In conclusion, our results indicated that intestinal bacteria from adults are able to degrade CML under anaerobic conditions. This study not only provides a better understanding of CML degradation by gut bacteria but also enables further studies on identifying enzymes that are responsible for this degradation and factors that might have an influence on the degradation. Even if studies on larger population are required, these results paved the way to introduce a closer view on the relationship between diet and CML metabolic fate revealing that also human microbiota is able to generate metabolites with a potential impact on pathophysiological outcomes connected to d-AGEs.

Abbreviations used

Dietary advanced glycation end-products, dAGES; *N*-ε-carboxymethyllysine, CML; dietary *N*-ε-carboxymethyllysine, d-CML; *N*-carboxymethylcadaverine, CM-CAD; *N*-carboxymethylaminopentanoic acid, CM-APA; hydrophilic interaction liquid chromatography, HILIC; liquid chromatography tandem mass spectrometry, LC-MS/MS; high resolution mass spectrometry, HRMS.

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Notes

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FIGURE CAPTIONS.

Figure 1: Microbial composition of the first and second enrichments in CML (A); CML usage in the first and second enrichment from sample AF1A (B) and morphology of *Oscillibacter* spp. observed in the enrichment (C). The incubation time was 5 weeks at 37 ^{0} C under anoxic conditions.

Figure 2: Microbial composition of the second transfer of CML enrichment from AS1 (A) and the CML usage in the first and second transfer (B). The incubation time was 5 weeks at 37 ^{0} C under anoxic conditions.

Figure 3: CML degradation by strain AS3 (A) and morphology of strain AS3 (B). AA_CML indicates a condition that an amino acid mixture was added along with CML while the other only contained CML as sole carbon and energy source.

Figure 4: Formation of CML degradation products during the anaerobic incubation of CML by *C. evryensis* strain AS3. CM-CAD (carboxymethyl-cadaverine); CM-APA (carboxymethyl-amino pentanoic acid). The label AA indicates the presence of other carbon source along with CML.

Figure 5: Hypothesized pathways for CML degradation according to KEGG and lysine metabolism of AS3 strains

Supplementary Figure 1: Phylogenetic tree based on 16S rRNA sequences obtained from the second transfer of CML enrichment from AS1 and closely related species.

Table 1: Volunteer Information

Subject	Origin	Code	Inoculum
Formula-fed infant	Netherlands	F1	Fresh
Breast-fed infant	Netherlands	M1	Fresh
Breast-fed infant	Netherlands	M2	Fresh
Adult	Asian	AS1	Fresh
Adult	African	AF1A	Frozen
Adult	African	AF4A	Frozen

Table 2: CML degradation in the first enrichments from 6 volunteers**.** The measurement was performed after 3 weeks incubation (*) or 5 weeks incubation.

	Samples					
CML concentration (mM)	F1*	AF4A*	M1*	AF1A	AS1	M2
T0	6.30	7.90	4.84	4.50	2.19	2.14
After 3w*/5w	5.13	7.47	3.84	1.00	0.00	2.11
Consumed (mM)	1.17	0.43	1.00	3.50	2.19	0.03
Consumed (%)	18.62	5.46	20.65	77.69	100.00	1.39

Table 3: Zwitterionic HILIC high-resolution mass spectrometry (HRMS) analysis. Error (Δ ppm) was calculated as the ratio between the difference of the theoretical mass minus the experimental mass and the theoretical mass, multiplied per one million. Theoretical mass (*m/z* T), experimental mass (*m/z* E), retention time (RT, min), elemental composition (EC) . *N*-carboxymethylcadaverine (CM-CAD), *N*-carboxymethylaminopentanoic acid (CM-APA).

Compound Name	**RT**	**EC**	***m/z T***	***m/z E***	Δ ppm
CM-CAD	2.8	C7H16N2O2	161.12845	161.12822	-1.4
CM-APA	3.1	C7H13NO4	176.09173	176.09201	1.6
2-amino-6-(formylmethylamino)hexanoic acid	3.5	C8H16N2O3	189.09955	189.09899	-3.0
N-carboxymethyl-Δ^1-piperideinium ion	3.6	$C7H12NO2^+$	142.08626	142.08599	-1.9

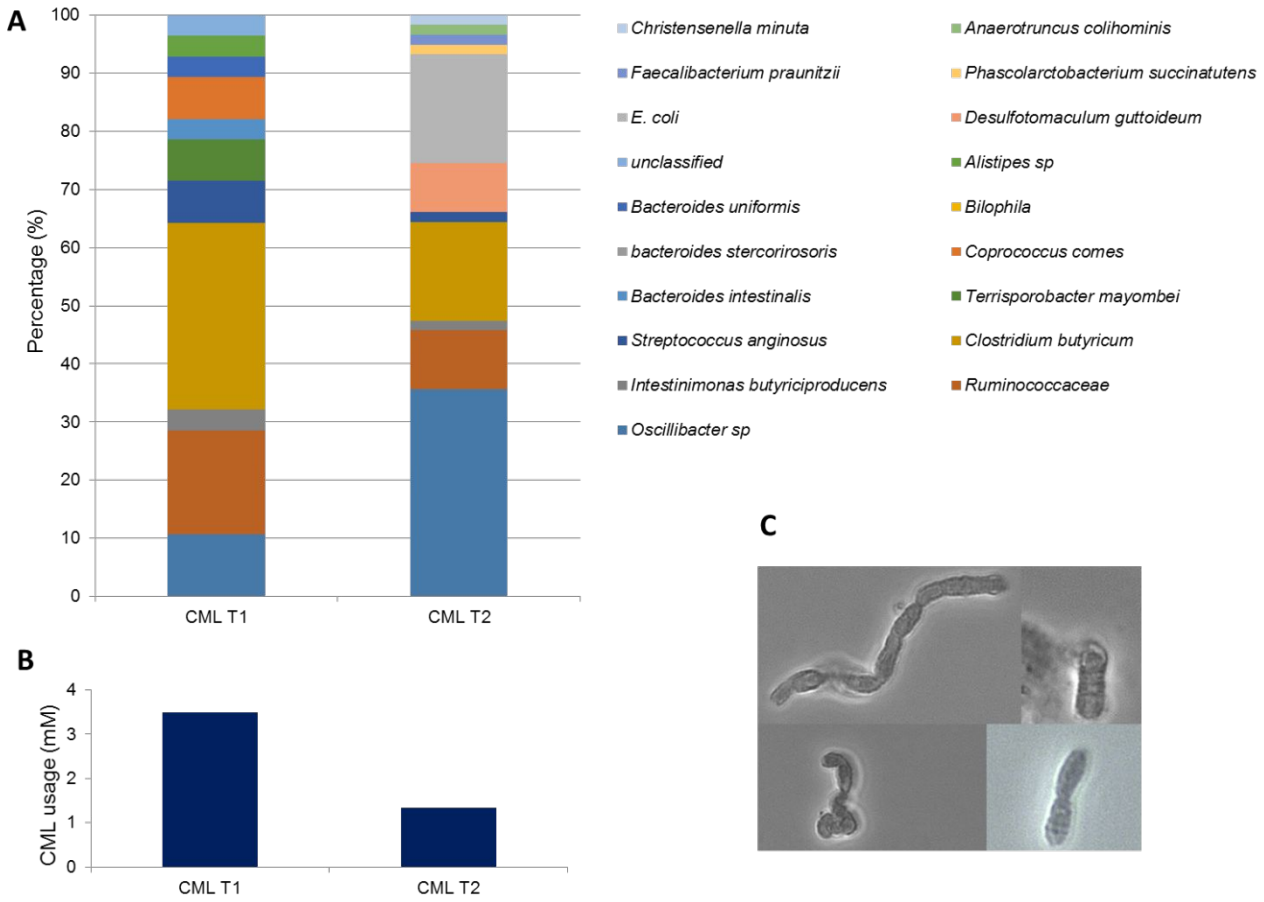

A
Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
CML T1
CML T2
Christensenella minuta
Anaerotruncus colihominis
Faecalibacterium praunitzii
Phascolarctobacterium succinatutens
E. coli
Desulfotomaculum guttoideum
unclassified
Alistipes sp
Bacteroides uniformis
Bilophila
bacteroides stercorirosoris
Coprococcus comes
Bacteroides intestinalis
Terrisporobacter mayombei
Streptococcus anginosus
Clostridium butyricum
Intestinimonas butyriciproducens
Ruminococcaceae
Oscillibacter sp
B
CML usage (mM)
4
3
2
1
0
CML T1
CML T2
C

Figure 1

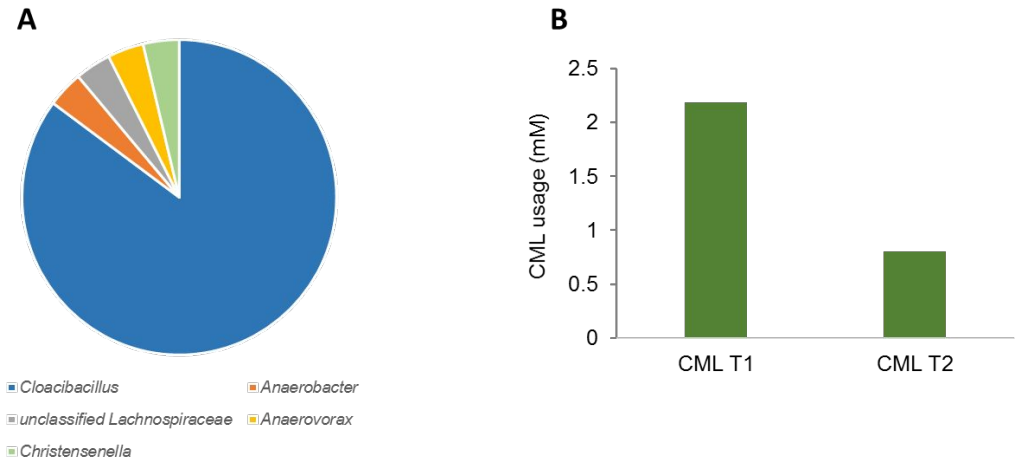

A
Cloacibacillus
Anaerobacter
unclassified Lachnospiraceae
Anaerovorax
Christensenella
B
CML usage (mM)
2.5
2
1.5
1
0.5
0
CML T1
CML T2

Figure 2

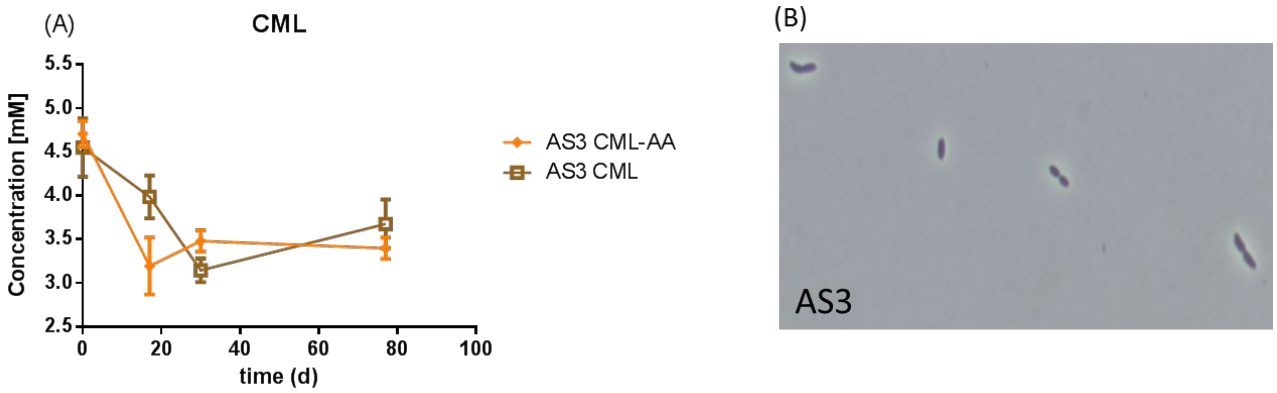

(A)
CML
Concentration [mM]
5.5
5.0
4.5
4.0
3.5
3.0
2.5
0
20
40
60
80
100
time (d)
AS3 CML-AA
AS3 CML
(B)
AS3

Figure 3

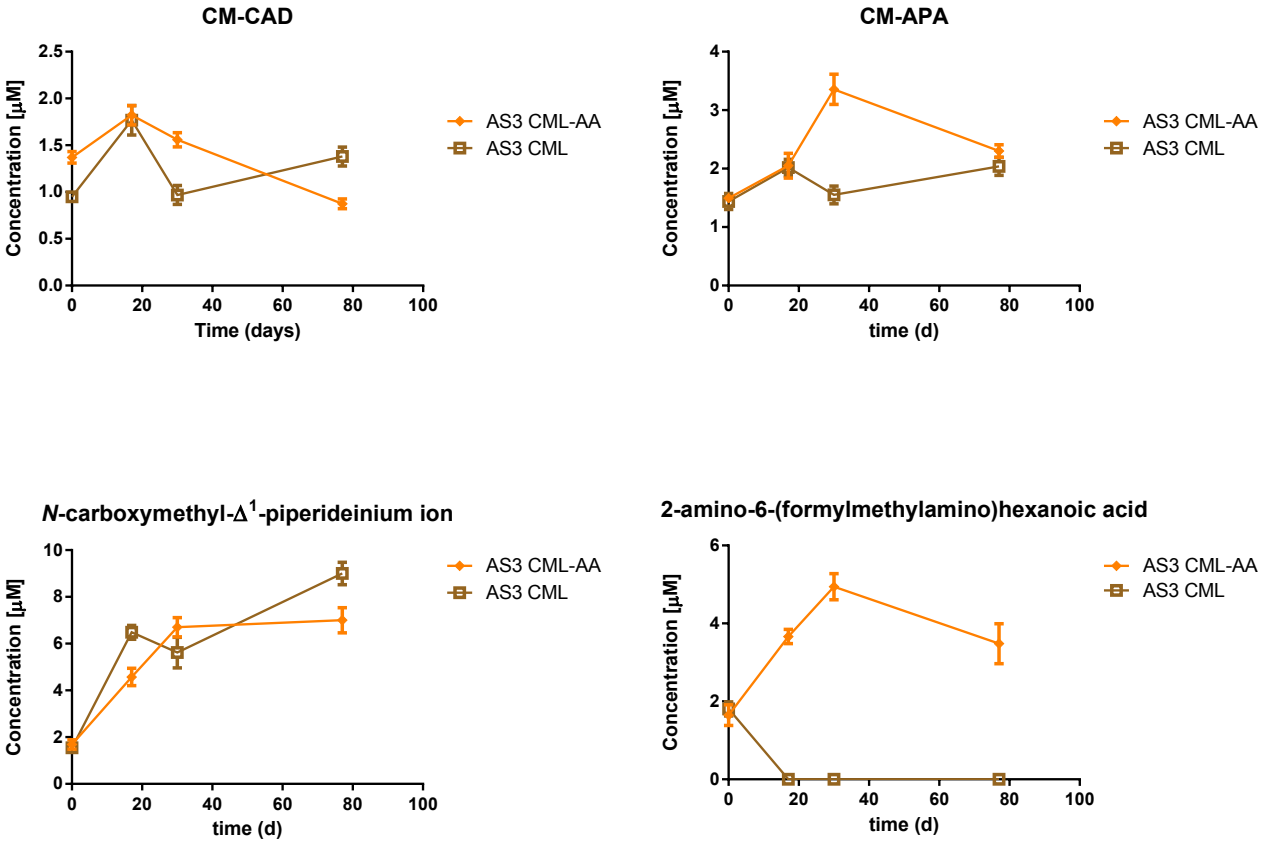

CM-CAD
Concentration [µM]
Time (days)
AS3 CML-AA
AS3 CML
CM-APA
Concentration [µM]
time (d)
AS3 CML-AA
AS3 CML
N-carboxymethyl-Δ1-piperideinium ion
Concentration [µM]
time (d)
AS3 CML-AA
AS3 CML
2-amino-6-(formylmethylamino)hexanoic acid
Concentration [µM]
time (d)
AS3 CML-AA
AS3 CML

Figure 4

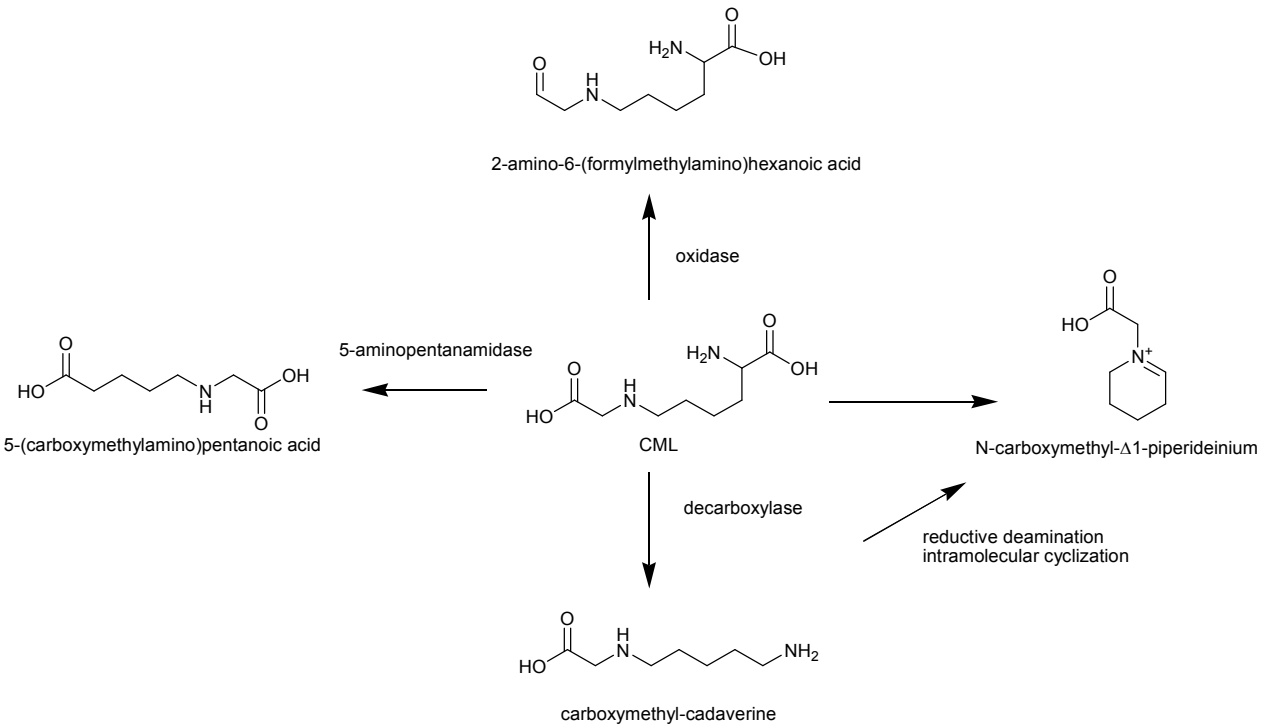
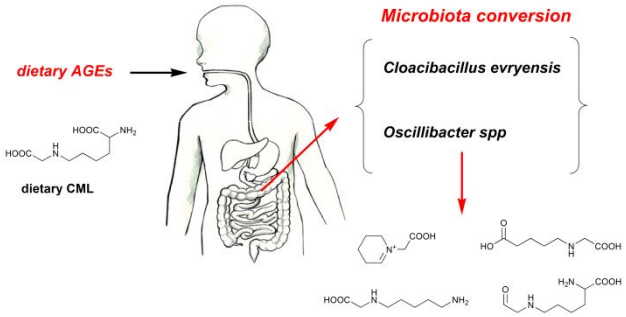

2-amino-6-(formylmethylamino)hexanoic acid
oxidase
5-aminopentanamidase
5-(carboxymethylamino)pentanoic acid
CML
N-carboxymethyl-Δ1-piperideinium
decarboxylase
reductive deamination
intramolecular cyclization
carboxymethyl-cadaverine

Figure 5

TOC Graphic


Microbiota conversion
dietary AGEs
Cloacibacillus evryensis
Oscillibacter spp
dietary CML