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Prebiotics and age, but not probiotics affect the transformation of 2-amino-3-methyl-3H-imidazo[4,5-*f*]quinoline (IQ) by fecal microbiota – An *in vitro* study

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ABSTRACT

Heterocyclic aromatic amines (HAAs) are carcinogens which are formed in meat cooked using high-temperature methods. The human gastrointestinal (GI) microbiota plays a crucial role in maintaining health in humans of different ages, and especially in the elderly. However, the GI microbiota, whose metabolism and composition changes with age, may also be responsible for the activation of mutagenic substances reaching the colon with diet. Probiotics and prebiotics are promising in terms of reducing the destructive effects of HAAs. The aim of the study was to determine if fecal microbiota derived from the feces of 27 volunteers: infants (up to 18 months), adults (aged 23–39 years), the sub-elderly (aged 64–65 years), and the elderly (aged 76–87 years), and the presence of probiotics or prebiotics, affected the transformation of IQ (2-amino-3-methylimidazo[4,5-*f*]quinoline) to 7-OH-IQ (2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-*f*]quinoline-7-one). The compounds were identified using LC-MS^n, NMR, and FTIR. Their genotoxicity was compared in the comet assay. Individual strains capable of IQ transformation were also identified. 7-OH-IQ was detected in six persons (two children and four elderly individuals). The degree of IQ conversion ranged from 26% (4-month-old girl) to 94% (81-year-old woman) of the initial quantity. Four *Enterococcus* isolates: two *Enterococcus faecium* and two *Enterococcus faecalis* strains, as well as one *Clostridium difficile* strain (LOCK 1030, from the culture collection) converted IQ to 7-OH-IQ. The genotoxicity of samples containing 7-OH-IQ was even three times higher ($P < 0.05$) than those with IQ and was correlated with the degree of IQ conversion and 7-OH-IQ concentration.

1. Introduction

Heterocyclic aromatic amines (HAAs) are produced during the Maillard reaction when meat is cooked at high temperatures (above 200 °C) or for prolonged periods of time at lower temperatures [1]. The highest quantities of HAAs have been detected in lean beef, chicken, and fish [1–3]. The preparation method plays a crucial role in the synthesis of HAAs, and especially grilling and frying enhance their formation [4]. 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) is one of the most common HAAs present in processed food (for the first time they were isolated from sun-dried sardines) and its occurrence is the highest in fried and broiled beef, also ground, as well as in fried fish and eggs [5]. It is also present in tobacco smoke [6,7]. The International Agency for Research on Cancer (IARC) classifies IQ as a probable human carcinogen (class 2A). IQ is mutagenic in the Ames test; in mammalian cells it induces sister chromatid exchange, strand breaks, and unscheduled DNA synthesis. It also induces tumors in the liver, intestines, lungs, and many other organs in rodents; the exposure of humans is associated with cancers of the pancreas, colon, prostate, and breasts [5,7].

The harmful effects of HAAs are connected to their metabolism in the human GI tract, which involves enzymes responsible for their activation or detoxification. In the human body, IQ can undergo

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oxidation, hydroxylation, nitrosation, and glucuronidation to genotoxic derivatives and DNA adducts, such as *N*-hydroxy-IQ, *N*-acetoxy-IQ and *N*-(deoxyguanosin-8-*yl*)-IQ [6–8]. More than 90% of ingested HAAs are removed from the jejunal and ileal segments of the human gastrointestinal tract, which means that nearly 10% of consumed HAAs reach the colon and get in touch with its microbiota [9]. The colon microbiota can be involved in the formation or activation of genotoxic, mutagenic and carcinogenic compounds from the pre-mutagenic substances that are delivered to the colon with diet. There are few reports concerning the metabolism of HAAs by the human colon microbiota. The main IQ metabolite assessed *in vitro* and *in vivo* in human flora associated (HMF) rats is 2-amino-3,6-dihydro-3-methyl-7H-imidazo[4,5-*f*]quinoline-7-one (7-OH-IQ) [10–12], which is a directly acting mutagen in the Ames test [13].

Humans are constantly exposed to HAAs [1]. Nowadays, considerable efforts are under way to identify food ingredients that could inhibit the harmful activity of HAAs, with a focus on probiotics and prebiotics. Probiotics are live microorganisms (mostly *Lactobacillus* and *Bifidobacterium* species) which, if administered in adequate amounts, confer a health benefit on the host [14]. In turn, prebiotics are defined as non-digestible food components which, if consumed in sufficient amounts, selectively stimulate the growth and activity of beneficial microbes in the colon, also resulting in health benefits [15]. Probiotics, prebiotics, and synbiotics (combinations of the former two) may have protective effects against the early stages of colon cancer. Their mechanisms of action may involve: altering the intestinal environment by reducing pH, lowering the activity of bacterial enzymes (*e.g.*, β-glucuronidase), enhancing short chain fatty acid production, increasing antiproliferative activity, promoting the apoptosis of cancer cells, stimulating immunomodulation, as well as binding and inactivating mutagenic and genotoxic compounds that are delivered to the colon with food, arise from internal metabolism, or are attributable to the exogenous environment [16,17].

There are no data on the influence of prebiotics, probiotics, and the fecal microbiota of persons of different ages on the generation of 7-OH-IQ. The aim of the presented study was to determine whether age and the presence of probiotic strains or prebiotics has an effect on the synthesis of 7-OH-IQ by fecal microbiota. To simulate *in vivo* conditions, human feces from infants, adults, and the elderly were collected and used, also in the form of an extract (fecal water, FW). IQ was incubated with human feces and its derivative was determined by LC-MS^n, NMR, and FTIR analysis. In subsequent experiments, individual bacterial strains metabolizing IQ were isolated from human feces and identified. Additionally, some collection strains of fecal bacteria originating from humans of different ages were examined for the ability to convert IQ. Finally, 7-OH-IQ genotoxicity was evaluated in the alkaline comet assay in comparison to IQ, using Caco-2 cells derived from the human colon carcinoma. This is also the first report of using FTIR for 7-OH-IQ analysis.

2. Materials and methods

2.1. Carcinogen

The heterocyclic aromatic amine IQ (2-amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline) was purchased from Toronto Research Chemicals, Canada. To obtain a stock solution, it was diluted in DMSO (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.25%. The stock solution was stored at 4 °C.

2.2. Collection and preparation of fecal samples

Feces were obtained from 27 healthy, non-smoking individuals without a history of GI disorders, who had not received antibiotics, probiotics and prebiotics during three months, and grilled products during fourteen days, prior to sample delivery. The subjects were divided into three main groups: nine infants from 4 to 18 months of age (four girls and five boys, all breast-fed, some were eating typical baby food according to nutritional requirements, at around 6 months of life), eight adults aged 23–28 (six women and two men), two sub-elderly individuals 64 and 66 years of age (one woman and one man), and eight elderly individuals aged 76–87 (one man and seven women). Seniors and adults were on a Western-type diet, without prevalence of some group of food (meats or vegetables). They were not vegetarians or vegans.

Morning fecal sample were collected into sterile plastic containers, transported at 4 °C under anaerobic conditions, and taken to the laboratory within 2–3 h after collection. To give a true picture of microbe profiles, participants (or their mothers in case of infants) were instructed to collect entire bowel movement. Fresh feces from each individual (whole sample) were supplemented with sterile phosphate-buffered saline (PBS, pH 7.0) (20%/80%, v/v) and homogenized in a stomacher for 2 min. Subsequently, IQ was added at a final concentration of 252 μM. To exclude the presence of IQ and its metabolite attributable to human metabolism, negative controls of feces from each individual in sterile PBS were prepared without the addition of IQ. Homogenized samples were sealed with butyl rubber tops and incubated at 37 °C for 72 h under anaerobic conditions. For chromatographic analysis, the samples were centrifuged (10,700 × *g*, 40 min, 4 °C) and 10 mL of each sample was filtered (0.45 μm pore size Syringe filters, Membrane Solutions, USA). The supernatant fractions (FW) were stored at −20 °C until subsequent analysis with LC-MS^n and NMR.

2.3. Chemical analysis

2.3.1. Solid phase extraction (SPE)

For qualitative and quantitative analysis of IQ and its metabolite, 7-OH-IQ, the compounds were extracted from all samples by performing SPE with Chromabond C18 cartridges (Macherey-Nagel, Germany). A Thermo Scientific 16-Port SPE Vacuum Manifold was used for SPE procedures. After centrifugation of fecal samples (as described in Section 2.2.), the resulting supernatants were loaded on 500 mg C18 cartridges preconditioned with 1 mL of methanol and 1 mL of deionized water. Next, 5 mL of a sample was added to the cartridge and washed with 1 mL of deionized water. Then, the analyte was eluted with 1 mL of 70% methanol, transferred into vials, and analyzed immediately.

2.3.2. LC-MS^n analysis

A HPLC system equipped with a PDA Accela absorbance detector (Thermo Scientific, Waltham, MA, USA) was coupled on-line with an MS LTQ Velos mass spectrometer (ThermoScientific, Waltham, MA, USA). Chromatographic separation was performed using a column operated at 25 °C. Samples (10 μL) were injected using an Accela Autosampler onto a Zorbax 300SB-C3 (Solvent Saver Plus, Agilent Technologies, USA) column (150 × 3 mm I.D., 3.5 μm). The mobile phase consisted of acetonitrile 90% (A) and formic acid 0.01% (B), and the flow rate was set to 0.3 mL/min.

Elution began with 2% A for 2 min, and then proceeded from 2% to 80% A for 18 min, from 80% to 100% A for 1 min, followed by washing and re-equilibration of the column. Mass spectra were recorded during 21 min. The absorbance of IQ and 7-OH-IQ was measured at 264 and 315 nm. Under these conditions, IQ was eluted at 7.93–8.37 min and 7-OH-IQ at 8.22–8.58 min.