Zamorano University

Food Science and Technology Department

B.S. in Food Science and Technology

Special Graduation Project

Influence of condensed tannins on in vitro fermentation of maize and potato starches using diverse human microbiomes

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Abstract

Condensed tannins are non-hydrolyzable polyphenols with well-documented effects on the gut microbiome, including stimulation of beneficial bacteria such as Faecalibacterium. This study evaluated the impact of tannin supplementation on the in vitro fermentation of various starch substrates, both purified (high-amylose maize, corn starch, potato starch) and whole grain flours (K55, K55AE, and Waxy maize), using fecal samples from nine individual human microbiomes.

After 16 hours of anaerobic fermentation, short-chain fatty acid (SCFA) concentrations and Faecalibacterium duncaniae growth were measured. Tannins consistently decreased butyrate and acetate production across treatments, while propionate remained relatively stable. Notably, the growth of Faecalibacterium was enhanced in whole grain treatments with tannins, while in the case of purified starches, the tannins negatively affected the microbial growth, resulting in reduced growth of Faecalibacterium duncaniae. These results indicate that the microbial response to tannins is strongly influenced by the food matrix, likely due to physical and chemical interactions with proteins and fibers that modulate tannin bioactivity.

These findings provide valuable insights into how dietary components, such as tannins, influence the composition and function of the gut microbiome. Moreover, these findings have implications for the design of functional foods that promote gut health by modulating the microbiome. This study contributes to the understanding of diet-microbiome interactions and supports the development of strategies to design foods that optimize human gut health.

Keywords: Polyphenols, butyrate production, dietary fibers, maize flours, phenolic metabolites, human gut fermentation.

Resumen

Los taninos condensados son polifenoles no hidrolizables con efectos bien documentados sobre el microbioma intestinal, incluyendo la estimulación de bacterias benéficas como Faecalibacterium. Este estudio evaluó el impacto de la suplementación con taninos en la fermentación in vitro de distintos sustratos de almidón, tanto purificados (maíz alto en amilosa, almidón de maíz y papa) como harinas de grano entero (K55, K55AE y maíz ceroso), utilizando inóculos fecales de nueve microbiomas humanos individuales.

Tras 16 horas de fermentación anaeróbica, se cuantificaron los ácidos grasos de cadena corta (AGCC) y el crecimiento de Faecalibacterium duncaniae mediante qPCR. La presencia de taninos redujo de forma consistente la producción de butirato y acetato, mientras que el propionato se mantuvo relativamente estable. Cabe destacar que el crecimiento de *Faecalibacterium* se vio favorecido en los tratamientos con granos enteros y taninos, mientras que en el caso de los almidones purificados, los taninos afectaron negativamente el crecimiento microbiano, lo que resultó en una reducción del crecimiento de *Faecalibacterium duncaniae*. Estos resultados indican que la respuesta microbiana a los taninos está fuertemente influenciada por la matriz alimentaria, probablemente debido a interacciones físicas y químicas con proteínas y fibras que modifican su bioactividad.

Los resultados obtenidos proporcionan una mejor comprensión de cómo los componentes dietéticos, como los taninos, influyen en la composición y función del microbioma intestinal. Además, estos hallazgos tienen implicaciones para el diseño de alimentos funcionales que promuevan la salud intestinal mediante la modulación del microbioma.

Palabras claves: Polifenoles, producción de butirato, fibra dietética, harina de maíz, metabolitos fenólicos, fermentación intestinal humana.

Introduction

The microbiome encompasses the diverse community of microorganisms, including bacteria, fungi, viruses, and their genetic material, that naturally colonize the human body. Despite their microscopic size, these microorganisms play a fundamental role in maintaining human health by contributing to metabolic processes, immune regulation, and overall homeostasis. The human microbiome is formed during the first years of life and can develop depending on the experiences and environment in which we live homeostasis.

Many factors such as our individual genetics and exposures to antibiotics or anti-microbial components can influence the composition of the microbiome, but diet is one of the most significant factors that can species composition and function of the microbiome. Among the dietary components that are known to have desirable effects on microbiome, resistant starches are probably the best studied. Resistant starch refers to the starch components that are resistant to digestion and pass undigested into the large intestine, where they are metabolized by the gut microbiome. Resistant starches are also known for their beneficial effects on disease prevention, including diabetes and obesity. However, detailed studies of the effects of RS in gnotobiotic mouse models demonstrated that its effects can be mediated through the microbiome as well as independent of the microbiome.

Starches exist with varying degrees of digestibility and fermentation potential, depending on the plant species from which they are derived, the genetic background of the plant species, and the method in which they are extracted or prepared. In general, starches are made up of the highly digestible and highly branched glucose polymer called amylopectin and the straight-chain, digestion-resistant glucose polymer amylose. Ratios of amylose/amylopectin vary in starches derived from different plant sources. For example, regular corn starch typically consists of 75% amylopectin and 25% amylose, while potato starch contains 11.9% to 20.1% amylose.

In addition to inter-species variation in starch composition, there are also naturally occurring mutations that have been found in all cereal grains that can affect amylose/amylopectin ratios. In maize, mutations giving rise to a waxy phenotype were discovered in the 1930s and later mapped to the Granule-Bound Starch Synthase (GBSS) which is responsible for synthesis of amylose. Waxy starches are generally >90% amylopectin and have unique functional properties such as gelling capacity that make them desirable in some food products. A second set of naturally occurring mutations in maize called “amylose-extender (AE)” affect starch composition by inactivating the starch branching enzyme (SBEIIb). This causes significant increases in the amylose content (and corresponding decrease in the amylopectin content), leading to the high-amylose or AE maize starch that is typically 65-80% amylose.

The waxy and amylose extender traits have been bred into several different genetic backgrounds, including a common parental line called K55. From the K55 parent, a set of three near-isogenic lines are available which includes the K55 parent, a K55-waxy (K55-WX) derivative, and a K55-amylose extender (K55AE) derivative. Together these three lines produce grain with different “doses” of amylose (K55-WX is <5% amylose, K55 is ~25% amylose and K55-AE is ~65% amylose), which enables dose-response type of experimental designs to study outcomes related to nutrition and human health in the context of a whole grain.

Amylose is a significant component of resistant starch (RS), which is the starch form that is resistant to digestion by host enzymes and passes intact into the colon, where it is fermented by the colonic microbiome to short chain fatty acids (SCFA). SCFA production is essential for gut health, as these metabolites exhibit anti-inflammatory properties, regulate immune responses, and protect against inflammatory bowel diseases. Additionally, substrates that can be fermented by the microbiome to SCFAs support a balanced microbial community, preventing dysbiosis by fostering the growth of beneficial RS-fermenting bacteria such as *Faecalibacterium*.

Faecalibacterium is one of the most abundant anaerobic bacteria in the human gut microbiota, contributing to gut health by consuming acetate and producing butyrate, along with bioactive anti-inflammatory molecules such as shikimic and salicylic acids.

Beyond fermentable carbohydrates, dietary polyphenols, such as tannins, also influence gut microbiota. Tannins are metabolized by gut microbes into bioactive compounds.

While their primary role is to modulate microbial composition, certain bacterial species can further process these metabolites, contributing to the production of SCFAs like acetate and butyrate, which support gut health. These bioactive metabolites also exhibit antioxidant, anti-inflammatory, and antimicrobial properties, shaping gut microbial function.

Tannins are a broad range of molecules comprising two major families, digestible and undigestible. Condensed tannins (CT) are considered undigestible and are made up of polymerized catechin ring structures. Production of CTs is believed to be an ancestral trait that was bred out during domestication of many of the cereal grains, due to the bitter flavors that they can impart. Among the cultivated varieties of cereal grains, CT production is only found in Sorghum and Millets, and the trait is highly variable across different cultivated lines. In addition to bitter flavors, CTs are also recognized for their anti-nutritional properties, which is generally due to the ability of CTs to bind a wide variety of proteins and starches, which reduces their bioavailability to the host.

The relationship between diet and the gut microbiome is fundamental to human health. What individuals consume not only nourishes them but also feeds the bacteria inhabiting their gut, influencing their balance and the production of essential compounds such as short-chain fatty acids. However, many questions remain regarding how distinct types of starches and compounds such as tannins affect this process. Therefore, this study aims to understand how starch fermentation occurs in the presence and absence of tannins, examining their impact on bacterial composition and SCFA production. Specifically, the objectives of this research are to evaluate the effect of tannins on fermentation and their interaction with different starch substrates on the growth and activity of *Faecalibacterium* in individual microbiomes, quantify the impact of tannins on short-chain fatty acid production during resistant starch fermentation, and compare the influence of starch type during fermentation modulation and the proliferation of *Faecalibacterium*.

Methodology

In vitro Digestion

The study began with the preparation of samples following the established procedure, where three types of corn grains (K55, K55AE, and Waxy) were processed using the Geno/Grinder 2025 (SPEX SamplePrep) at 1600 rpm for 10 min to obtain fine powder. Three replicates were prepared for each sample. Additional substrates, including High amylose, potato starch, and Corn starch, were also included.

Then 2.5 g of each sample was mixed with 30 mL of distilled water in 50 mL Falcon tubes (since potato and regular corn starch tended to gelatinize, they were pre-treated by diluting them with 120 mL and 60 mL of sterile water, respectively). These tubes were placed on a shaker at 200 rpm for 20 minutes at room temperature. Subsequently, the samples underwent a gelatinization step, during which they were submerged in boiling water with intermittent agitation every 30 seconds for 10 minutes, followed by an additional 10 minutes of cooking. The tubes were then transferred to an orbital shaker and incubated at 37°C for 40 minutes at 200 rpm.

Enzymatic Digestions

Enzymatic digestion was carried out in two main phases. The first phase involved acid digestion, where the pH was reduced to ~2.5 by adding 0.8 mL of 1 M HCl, followed by the addition of 1 mL of pepsin solution (100 mg/mL in 50 mM HCl, P-7000, Sigma, St. Louis, MO, USA). The tubes were incubated at 37°C with continuous agitation (200 rpm) for 1 hour.

In the second phase, enzymatic hydrolysis was performed. The pH increased to ~6.9 by adding 1.6 mL of 0.5 M $NaHCO_3$. Subsequently, 5 mL of pancreatin (125 mg/mL, P-7545, Sigma, St. Louis, MO, USA) in 0.1 M sodium maleate buffer and 0.2 mL of amyloglucosidase (3260 U/mL, E-AMG, Megazyme) were introduced. This mixture was incubated at 37°C under shaking conditions (200 rpm) for 6 hours.

The samples then underwent dialysis to remove smaller molecular weight compounds. Dialysis membranes (molecular weight cutoff of 1000 Dalton) were pre-soaked in RO water for 30 minutes to eliminate preservatives and thoroughly rinsed. The digested contents were transferred into the membranes, with additional rinses using RO water. The dialysis process was conducted at 4°C in 5 gallons of distilled water, with water changes every 12 hours for a total duration of 72 hours. Once dialysis was complete, the samples were transferred to 50 mL Falcon tubes and were frozen at -80°C before undergoing freeze-drying for three days.

Fecal Samples and In vitro Fermentation

Samples were acquired from nine healthy adults who had no history of gastrointestinal disorders and had not used any probiotics, prebiotics, or antibiotics in the previous six months. Fresh fecal samples were collected from enrolled and consented subjects meeting the inclusion criterion under UNL IRB (20240323184EP). Samples were collected using a commode collection kit (Fisher Scientific, NH, USA).

A 1:10 fecal suspension was prepared in an anaerobic chamber (5% H_2, 5% CO_2, and 90% N_2; Bactron X, Sheldon Manufacturing, Cornelius, OR, USA) within two hours of sample collection. The preparation involved mixing the sample with sterile 10% glycerol in phosphate-buffered saline (PBS) at pH 7.0 in a 1:9 (w/v) ratio. The mixture was homogenized using a stomacher for 4 minutes and subsequently stored at -80°C until fermentation.

In vitro batch fermentations were conducted inside the anaerobic chamber. A total of 250 microliters of the resuspended sample were dispensed into a 1 mL deep well of a 96-well plate. An equal volume (250 µL) of 2X fermentation medium was added to each well. The fermentation medium, per liter, contained: 1 g Bacto casitone, 1 g yeast extract, 2 g K_2HPO_4, 3.2 g $NaHCO_3$, 3.5 g NaCl, 1 mL hemin solution (KOH 0.28 g, 95% ethanol 25 mL, hemin 100 mg, and ddH_2O to 100 mL), 0.05 g bile salts, 0.5 g/L cysteine HCl, 0.6 mL resazurin (0.1%), 10 mL ATCC trace mineral supplement, 3.6 mL volatile fatty acid (VFA) solution (17 mL acetic acid, 1 mL n-valeric acid, 1 mL iso-valeric acid, 1 mL iso-butyric acid, mixed with 20 mL of 10 mM NaOH), 10 mL ATCC vitamin supplement, and 1 mL vitamin K-3 solution (0.14 g vitamin K-3 in 100 mL of 95% ethanol) .

The commercial quebracho wood tannin extract (QT) was incorporated into the 2X fermentation medium at a concentration of 6.66 mg/mL after autoclaving. The medium was mixed with Oxyrase (0.1 mL/1 mL of medium). The prepared mixture was then added to the plate and left for 1 hour. Subsequently, 0.05 mL of fecal slurry was inoculated. In vitro fermentations were incubated at 37°C for 16 hours. Following fermentation, samples were centrifuged at 4000 g for 10 minutes. Both pellets and supernatants were stored at -80°C for further analysis.

DNA Extraction

The DNA was performed using the BioSprint 96 system, following the BioSprint® 96 One-For-All Vet Handbook protocol. First, the pellets from fermented samples were thawed and resuspended in ASL buffer before being transferred to a bead-beating plate. Mechanical disruption was conducted at 1800 rpm in cycles with intermediate resting periods, followed by enzymatic treatment with proteinase K and incubation at 70°C. Subsequently, the lysate was prepared, and the samples were processed using the BioSprint 96 automated system, which employed magnetic bead-based purification. The purified DNA was collected in an elution plate and stored at -20°C until further analysis. This DNA was then used for qPCR analysis to assess microbial population dynamics.

Faecallibacterium duncaniae quantification

The presence of *Faecallibacterium duncaniae* was quantified by quantitative polymerase chain reaction (qPCR) method using primers (Forward**:** CCATGAATTGCCTTCAAAACTGTT; Reverse**:** GAGCCTCAGCGTCAGTTGGT). Quantitative PCR reactions were prepared in duplicates with a total volume of 10 µL, containing 5 µL of 2× SYBR Green, 3 µL of nuclease-free water, 1 µL of primer mix (a combination of forward and reverse primers, each at 5 µM), and 1 µL of DNA template. The thermocycling conditions included: (i) an initial denaturation step for 5 minutes at 95 °C; (ii) 40 cycles consisting of 20 seconds at 95 °C, 25 seconds at an annealing temperature of 63 °C, and 30 seconds at 72 °C; (iii) one cycle of 15 seconds at 95 °C; (iv) one cycle of 30 seconds at 60 °C; (v) a 20-minute interval to generate the melting curve.

SCFA Analysis

Short-chain fatty acids (acetate, propionate, butyrate and valerate) and the branched fatty acids (isobutyrate and isovalerate) were extracted from the samples and analyzed using gas chromatography (GC) to determine fermentation end products, as previously described. The SCFA data was further processed to assess metabolic changes. In short, 100 µL aliquots of fermentation supernatant were combined with 100 µL of 7 nM 2-Ethylbutyric acid, 100 µL of 9 M sulfuric acid, and 500 µL of diethyl ether. The mixture was then homogenized using a vortex mixer and centrifuged for 2 minutes.

Experimental Desing

This study utilized a Randomized Complete Block Design (RCBD) to evaluate the effects of fermentation and the interaction between tannins and various starch substrates during the growth and activity of *Faecalibacterium* in individual microbiomes.

Treatments

The experimental treatments consisted of six different starch substrates, each tested with and without tannins, resulting in a total of 12 treatments.

Table 1

Treatments to evaluate with and without tannins added.

Starch Substrate	Tannin Presence	Treatment ID
K55 corn	With Tannins	K55_Tan
K55AE corn	With Tannins	K55AE_Tan
Waxy corn	With Tannins	Waxy_Tan
High amylose starch	With Tannins	HighA_Tan
Corn starch	With Tannins	CornStarch_Tan
Potato starch	With Tannins	Potato_Tan
K55 corn	Without Tannins	K55_No
K55AE corn	Without Tannins	K55AE_No
Waxy corn	Without Tannins	Waxy_No
High amylose starch	Without Tannins	HighA_No
Corn starch	Without Tannins	Corn_No
Potato starch	Without Tannins	Potato_No

Note. Each treatment was evaluated in triplicate (3 repetitions per treatment) to ensure statistical reliability.

Blocking Factor

To account for inter-individual microbiome variability, nine different microbiomes served as blocking factors. Each microbiome was exposed to all twelve treatments in a randomized manner.

Fermentation Setup and Microplate Distribution

Fermentation was conducted using four 96-well MAP plates, where each plate included the following controls and experimental wells

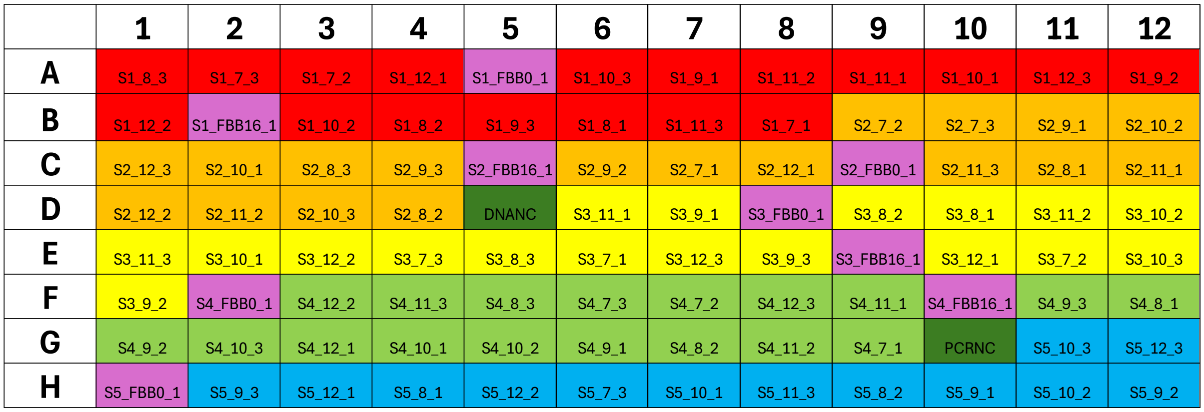
Controls (per plate).

3 wells for FBB16 (Post fermentation condition)

Each microbiome was randomly assigned across the plates to ensure balanced distribution. The layout of the plates was documented through a detailed schematic representation, illustrating the placement of microbiomes, treatments, and controls.

Figure 1

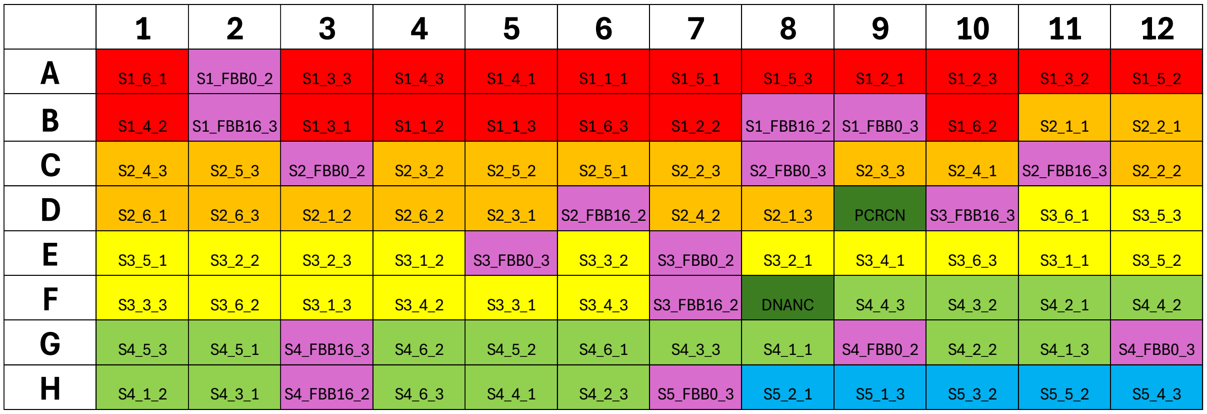
Map plate 1 (Substrates with tannins)



	1	2	3	4	5	6	7	8	9	10	11	12
A	S1_8_3	S1_7_3	S1_7_2	S1_12_1	S1_FBB0_1	S1_10_3	S1_9_1	S1_11_2	S1_11_1	S1_10_1	S1_12_3	S1_9_2
B	S1_12_2	S1_FBB16_1	S1_10_2	S1_8_2	S1_9_3	S1_8_1	S1_11_3	S1_7_1	S2_7_2	S2_7_3	S2_9_1	S2_10_2
C	S2_12_3	S2_10_1	S2_8_3	S2_9_3	S2_FBB16_1	S2_9_2	S2_7_1	S2_12_1	S2_FBB0_1	S2_11_3	S2_8_1	S2_11_1
D	S2_12_2	S2_11_2	S2_10_3	S2_8_2	DNANC	S3_11_1	S3_9_1	S3_FBB0_1	S3_8_2	S3_8_1	S3_11_2	S3_10_2
E	S3_11_3	S3_10_1	S3_12_2	S3_7_3	S3_8_3	S3_7_1	S3_12_3	S3_9_3	S3_FBB16_1	S3_12_1	S3_7_2	S3_10_3
F	S3_9_2	S4_FBB0_1	S4_12_2	S4_11_3	S4_8_3	S4_7_3	S4_7_2	S4_12_3	S4_11_1	S4_FBB16_1	S4_9_3	S4_8_1
G	S4_9_2	S4_10_3	S4_12_1	S4_10_1	S4_10_2	S4_9_1	S4_8_2	S4_11_2	S4_7_1	PCRNC	S5_10_3	S5_12_3
H	S5_FBB0_1	S5_9_3	S5_12_1	S5_8_1	S5_12_2	S5_7_3	S5_10_1	S5_11_3	S5_8_2	S5_9_1	S5_10_2	S5_9_2

Figure 2

Map plate 2 (Substrates without tannins)



	1	2	3	4	5	6	7	8	9	10	11	12
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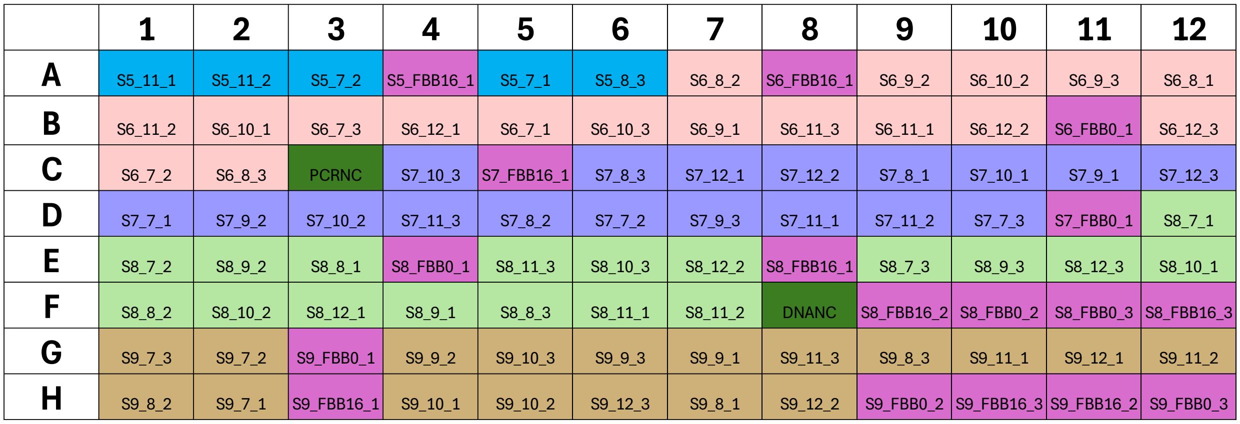
B	S1_4_2	S1_FBB16_3	S1_3_1	S1_1_2	S1_1_3	S1_6_3	S1_2_2	S1_FBB16_2	S1_FBB0_3	S1_6_2	S2_1_1	S2_2_1
C	S2_4_3	S2_5_3	S2_FBB0_2	S2_3_2	S2_5_2	S2_5_1	S2_2_3	S2_FBB0_3	S2_3_3	S2_4_1	S2_FBB16_3	S2_2_2
D	S2_6_1	S2_6_3	S2_1_2	S2_6_2	S2_3_1	S2_FBB16_2	S2_4_2	S2_1_3	PCRCN	S3_FBB16_3	S3_6_1	S3_5_3
E	S3_5_1	S3_2_2	S3_2_3	S3_1_2	S3_FBB0_3	S3_3_2	S3_FBB0_2	S3_2_1	S3_4_1	S3_6_3	S3_1_1	S3_5_2
F	S3_3_3	S3_6_2	S3_1_3	S3_4_2	S3_3_1	S3_4_3	S3_FBB16_2	DNANC	S4_4_3	S4_3_2	S4_2_1	S4_4_2
G	S4_5_3	S4_5_1	S4_FBB16_3	S4_6_2	S4_5_2	S4_6_1	S4_3_3	S4_1_1	S4_FBB0_2	S4_2_2	S4_1_3	S4_FBB0_3
H	S4_1_2	S4_3_1	S4_FBB16_2	S4_6_3	S4_4_1	S4_2_3	S5_FBB0_3	S5_2_1	S5_1_3	S5_3_2	S5_5_2	S5_4_3

Figure 3

Map plate 3 (Substrates with tannins)



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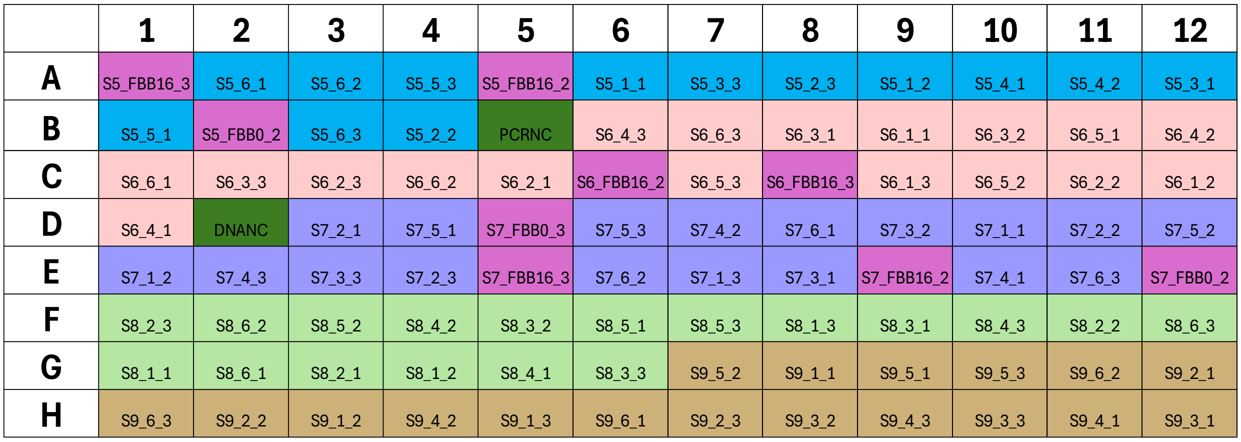
B	S6_11_2	S6_10_1	S6_7_3	S6_12_1	S6_7_1	S6_10_3	S6_9_1	S6_11_3	S6_11_1	S6_12_2	S6_FBB0_1	S6_12_3
C	S6_7_2	S6_8_3	PCRNC	S7_10_3	S7_FBB16_1	S7_8_3	S7_12_1	S7_12_2	S7_8_1	S7_10_1	S7_9_1	S7_12_3
D	S7_7_1	S7_9_2	S7_10_2	S7_11_3	S7_8_2	S7_7_2	S7_9_3	S7_11_1	S7_11_2	S7_7_3	S7_FBB0_1	S8_7_1
E	S8_7_2	S8_9_2	S8_8_1	S8_FBB0_1	S8_11_3	S8_10_3	S8_12_2	S8_FBB16_1	S8_7_3	S8_9_3	S8_12_3	S8_10_1
F	S8_8_2	S8_10_2	S8_12_1	S8_9_1	S8_8_3	S8_11_1	S8_11_2	DNANC	S8_FBB16_2	S8_FBB0_2	S8_FBB0_3	S8_FBB16_3
G	S9_7_3	S9_7_2	S9_FBB0_1	S9_9_2	S9_10_3	S9_9_3	S9_9_1	S9_11_3	S9_8_3	S9_11_1	S9_12_1	S9_11_2
H	S9_8_2	S9_7_1	S9_FBB16_1	S9_10_1	S9_10_2	S9_12_3	S9_8_1	S9_12_2	S9_FBB0_2	S9_FBB16_3	S9_FBB16_2	S9_FBB0_3

Figure 4

Map plate 5 (Substrates without tannins).



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B	S5_5_1	S5_FBB0_2	S5_6_3	S5_2_2	PCRNC	S6_4_3	S6_6_3	S6_3_1	S6_1_1	S6_3_2	S6_5_1	S6_4_2
C	S6_6_1	S6_3_3	S6_2_3	S6_6_2	S6_2_1	S6_FBB16_2	S6_5_3	S6_FBB16_3	S6_1_3	S6_5_2	S6_2_2	S6_1_2
D	S6_4_1	DNANC	S7_2_1	S7_5_1	S7_FBB0_3	S7_5_3	S7_4_2	S7_6_1	S7_3_2	S7_1_1	S7_2_2	S7_5_2
E	S7_1_2	S7_4_3	S7_3_3	S7_2_3	S7_FBB16_3	S7_6_2	S7_1_3	S7_3_1	S7_FBB16_2	S7_4_1	S7_6_3	S7_FBB0_2
F	S8_2_3	S8_6_2	S8_5_2	S8_4_2	S8_3_2	S8_5_1	S8_5_3	S8_1_3	S8_3_1	S8_4_3	S8_2_2	S8_6_3
G	S8_1_1	S8_6_1	S8_2_1	S8_1_2	S8_4_1	S8_3_3	S9_5_2	S9_1_1	S9_5_1	S9_5_3	S9_6_2	S9_2_1
H	S9_6_3	S9_2_2	S9_1_2	S9_4_2	S9_1_3	S9_6_1	S9_2_3	S9_3_2	S9_4_3	S9_3_3	S9_4_1	S9_3_1

Data Analysis

The impact of type and tannin presence on bacterial growth and metabolic activity was evaluated. To analyze the short-chain fatty acids (SCFAs), SAS (version 9.4) was used to compare treatments (with and without tannins) using two-way analysis of variance (ANOVA) and Duncan's test to identify significant differences.

Faecalibacterium quantification was based on CT values obtained through qPCR, which were converted to log (CFU) using a standard curve constructed via linear regression from a serial dilution of a known culture. Subsequently, log (CFU) values were compared across treatments (with and without tannins) using analysis of variance (ANOVA), followed by Duncan’s multiple range test to determine statistically significant differences between groups. All analyses and visualizations were performed using the Statistical Analysis System (SAS version 9.4)

Results and Discussion

This study aimed to evaluate the modulatory effect of tannin supplementation on the production of short-chain fatty acids (SCFAs) and the growth of *Faecalibacterium* during in vitro fermentation of different carbohydrate matrices, including whole grain flours and purified starches. Anaerobic fermentations were carried out for 16 hours using fecal inoculum from nine individual human gut microbiomes (S1–S9). SCFA concentrations were quantified in millimolar units (mM) as indicators of microbial metabolic activity, while bacterial growth was assessed as log CFU to estimate changes in population density. Experimental treatments included substrates both with and without added tannins, enabling a comparative analysis of their effects on microbial fermentation and metabolite output. Growth responses were analyzed individually for each microbiome and summarized as an overall mean trend. Statistical comparisons among treatments were performed using ANOVA and Duncan test separation to determine the significance of tannin effects across the tested matrices.

The human gut microbiome is a highly diverse and functionally dynamic ecosystem, predominantly composed of bacteria from the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria. Among the Firmicutes, *Faecalibacterium duncaniae* (formerly grouped under *F. prausnitzii)*, is a strictly anaerobic commonly found in the human gut. It produces butyrate, a short-chain fatty acid essential for colonocyte energy, gut barrier integrity, and immune regulation. Reduced levels have been linked to inflammatory bowel disease and metabolic disorders, highlighting its role as a marker of gut health.

Although *F. duncaniae* plays a central role in gut health, it lacks the enzymatic machinery to degrade complex polysaccharides directly. Instead, it thrives through cross-feeding interactions, utilizing metabolic products such as acetate, lactate, and oligosaccharides released by primary degraders like *Bacteroides thetaiotaomicron or Ruminococcus bromii.* This behavior is especially evident when fermentable substrates like resistant starch type 2 (RS2) are present, which enhance its growth and butyrate production in co-culture systems. Consequently, *F. duncaniae* contributes to gut homeostasis not by breaking down dietary fiber directly, but by efficiently converting intermediates into butyrate through the acetyl-CoA pathway, reinforcing its therapeutic relevance in diets rich in resistant starch and fermentable carbohydrates.

Short chain fatty acid production after 16 hours of fermentation

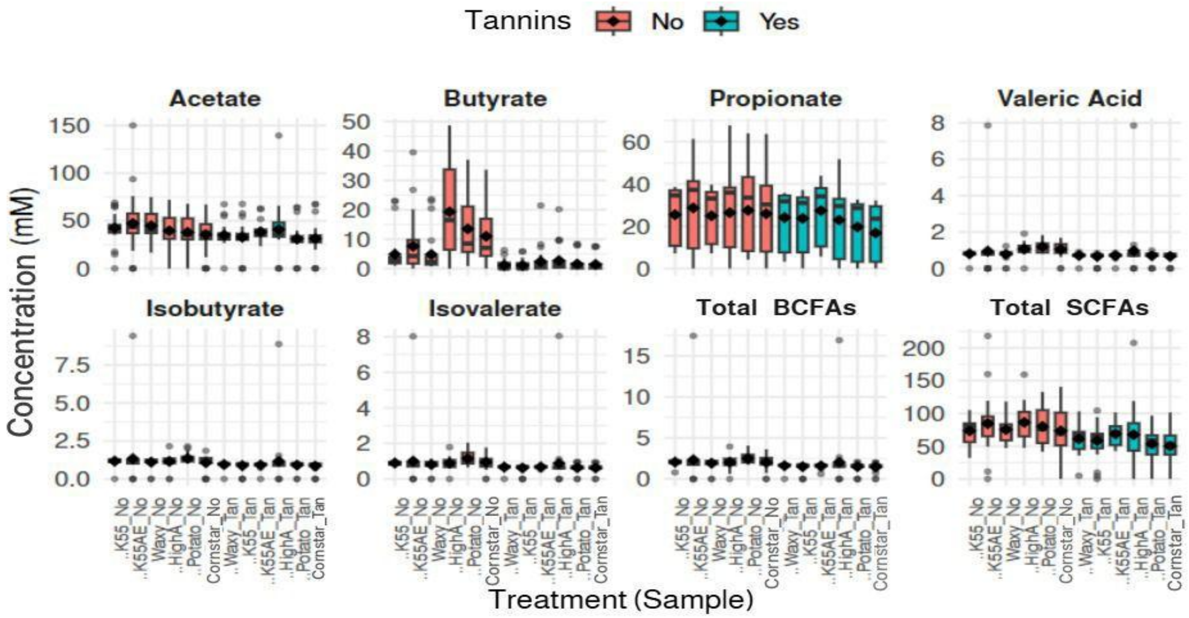
Tannin supplementation had a measurable impact on the overall production of short-chain fatty acids (SCFAs), as shown in Figure 5 Visual differences are most noticeable for butyrate, where concentrations appear lower in tannin-supplemented treatments. This trend is consistent with the statistical results summarized in Table 2, which report significant differences ($p < 0.0001$) for butyrate, particularly in substrates such as Waxy and Potato Starch.

Total SCFA concentrations follow a similar pattern, with several tannin-supplemented treatments showing reduced levels compared to their non treatment counterparts, Table 2 also reflects this tendency in acetate, where some substrates without tannins present higher mean values than those with tannins, although the differences are less pronounced than for butyrate.

In contrast, propionate levels appeared relatively stable across treatments in the Figure 5, and Table 2, shows that although there are statistically significant differences for propionate, these are comparatively smaller than those observed for butyrate and acetate. Branched-chain fatty acids (BCFAs), including isobutyrate and isovalerate, remain consistently low in all treatments according to Figure 5.

Figure 5

Concentrations of short and branched chain fatty acids (SCFAs and BCFAs) after 16 h fermentation across substrates with and without tannin supplementation, representing nine evaluated microbiomes.



Note: Tannin: presence or absence of tannins in the substrate. SCFAs: short-chain fatty acids. Pink: without tannins; blue: with tannins. Boxplots show median, interquartile range, and outliers.

The Table 2 displays the mean concentrations of acetate, propionate, and butyrate across different substrate treatments with and without tannin supplementation. Overall, tannin-free treatments consistently yielded higher SCFA concentrations, with the most pronounced effect observed for butyrate.

Acetate concentrations were significantly higher in tannin free treatments across all substrates. K55AE without tannins (47.26 mM ^{a}) and Waxy without tannins (44.92 mM ^{ab}) ranked among the highest groups, while their tannin supplemented counterparts shifted to lower groups (38.34 mM ^{abc} and 35.08 mM ^{bc}, respectively). Similar patterns were observed in Potato and Corn Starch, confirming that tannin presence consistently lowered acetate production.

In case of propionate concentrations showed statistically significant differences among treatments), however, Duncan's groupings indicate that most treatments, both with and without tannins, belong to overlapping groups (letters a-ab). Only a few treatments, such as Corn Starch with tannins (16.79

mM ^{b}), were clearly separated into lower groups. This indicates that while tannins exert some inhibitory effect on propionate, the response is moderate and varies depending on the substrate.

In contrast, butyrate showed the most pronounced response to tannin presence. All tannin supplemented treatments were grouped in the lowest category (letter e, ≤2.7 mM), while tannin-free treatments occupied higher groups (letters a-d) with concentrations up to 19.27 mM in High Amylose and 13.46 mM in Potato Starch.

The Duncan test confirms through means significantly different a consistent inhibitory effect of tannins on SCFA production, with the strongest impact on butyrate, moderate on acetate, and minimal on propionate. The coefficients of variation were 35.45% for acetate, 15.38% for propionate, and 37.44% for butyrate. The relatively high CVs for acetate and butyrate are attributed to differences in the individual gut microbiomes of the donors, which inherently vary in their taxonomic composition and metabolic capacity. This interpretation is supported by , who demonstrated that gut microbiota composition is a complex polygenic trait shaped by multiple host genetic and environmental factors, leading to substantial interindividual variability even under controlled conditions.

Table 22

Mean concentration of short chain fatty acids (acetate, propionate, and butyrate) under different substrate treatments with and without tannins

	Tannins	Substrate	Acetate Acid (Mm)	Propionate Acid (Mm)	Butyric Acid (Mm)
			Mean ± SE	Mean ± SE	Mean ± SE
Treatments	Yes	K55	32.88 ± 2.88 ^{c}	23.71 ± 2.60 ^{ab}	1.03 ± 0.32 ^{e}
		K55AE	38.34 ± 1.70 ^{abc}	27.38 ± 2.68 ^{a}	2.38 ± 0.85 ^{e}
		Waxy	35.08 ± 2.26 ^{bc}	24.10 ± 2.58 ^{ab}	1.05 ± 0.31 ^{e}
		Potato Starch	31.23 ± 2.94 ^{c}	19.67 ± 2.62 ^{ab}	1.55 ± 0.47 ^{e}
		Corn Starch	31.41 ± 3.13 ^{c}	16.79 ± 2.55 ^{b}	1.38 ± 0.43 ^{e}
		High Amylose	40.99 ± 5.06 ^{abc}	22.88 ± 2.96 ^{ab}	2.69 ± 0.87 ^{e}
	No	K55	42.37 ± 2.96 ^{b}	25.46 ± 2.52 ^{ab}	4.82 ± 1.25 ^{de}
		K55AE	47.26 ± 5.75 ^{a}	28.65 ± 3.32 ^{a}	7.58 ± 1.74 ^{cd}
		Waxy	44.92 ± 3.00 ^{ab}	24.89 ± 2.46 ^{ab}	4.78 ± 1.27 ^{de}
		Potato Starch	37.94 ± 4.00 ^{abc}	27.51 ± 3.81 ^{a}	13.46 ± 2.12 ^{b}
		Corn Starch	35.51 ± 3.78 ^{bc}	25.92 ± 3.85 ^{ab}	10.95 ± 1.94 ^{bc}
		High Amylose	39.57 ± 3.64 ^{abc}	26.42 ± 3.73 ^{ab}	19.27 ± 3.04 ^{a}
Pr > F			<0.0001	<0.0001	<0.0001
CV (%)			35.45	15.38	37.44

Note. Tanin: presence or absence of tannins in the substrate. SE: standard error. CV: coefficient of variation. Pr > F: probability value from ANOVA. Different letters indicate statistically significant differences between treatments within each column, according to Duncan's multiple range test (α = 0.05).

In contrast to the significant reductions observed for butyrate and acetate, propionate levels remained relatively stable across tannin treatments. This suggests that the microbial taxa responsible for propionate production may exhibit greater resilience to tannin induced inhibition. According to (2018) Bacteroides spp. are more commonly propiogenic than butyrogenic, and their presence was associated with elevated propionate molar ratios even when butyrate concentrations fluctuated. This could explain the maintenance of propionate output in treatments where Bacteroides remained functionally active despite tannin exposure.

Additionally, *Acidipropionibacterium acidipropionici*, another known propionate producing species, synthesizes this SCFA through two distinct metabolic routes: the Wood–Werkman cycle, which converts pyruvate into propionate while conserving redox balance, and the acrylate pathway, which uses lactate as an intermediate (Miyamoto et al., 2024). These complementary pathways give the bacterium metabolic flexibility, allowing it to maintain propionate synthesis under various environmental conditions.

This metabolic redundancy likely contributes to the observed stability of propionate levels under polyphenol-induced stress. Furthermore, Miyamoto et al. (2024) showed that SCFA production can remain functionally active even without major shifts in the composition of the microbiota, supporting the idea that metabolic function may be preserved despite tannin induced changes.

Nevertheless, the global reduction in butyrate and acetate levels supports previous findings that polyphenols can inhibit fermentative bacteria when present in high concentrations. Ozdal et al. (2016) highlighted that although low doses of polyphenols may stimulate beneficial microbes, higher levels often suppress microbial fermentation and decrease SCFA output (a pattern that aligns with the outcomes observed in our tannin-rich treatments).

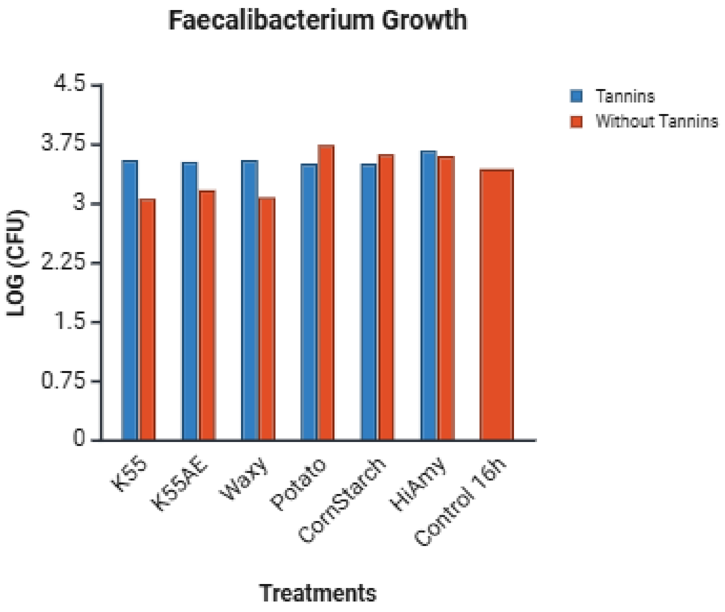
One possible mechanism contributing to the divergent effects observed among SCFAs may involve differential interactions between tannins and carbohydrate substrates. As noted by Kaur et al. (2025), tannins can form complexes with starches, particularly those rich in amylopectin, altering their physicochemical structure and reducing enzymatic digestibility. This could explain why the greatest reductions were observed in Waxy corn, Corn Starch, and Potato Starch, while resistant starches like High Amylose and K55AE, although still affected, retained comparatively higher SCFA levels.

Microbial growth of *Feacelibaterium* with and without tannins added

The Figure 6, shows the growth of *Faecalibacterium* (log CFU) after 16 hours of fermentation with and without tannins across different substrates. The control (FBB16), which lacked added substrates, showed bacterial counts around 3.2 log CFU. Whole grain-based treatments (K55, K55AE, Waxy) without tannins displayed values close to or slightly lower than the control, while the same substrates with tannins showed slightly higher values. Starch based treatments (Potato, Corn Starch, High Amylose) presented values similar to the control, with minimal differences between tannin-supplemented and non-supplemented conditions. Overall, bacterial counts across treatments remained within 3.0–4.0 log CFU, and statistical analysis indicated a significant treatment effect ($p < 0.0001$).

Figure 6

Microbial Growth of Faecalibacterium (log CFU) in Response to Tannin Addition Across Sample Types after 16 hours of fermentation.



Note. Tannins: Red = no tannins; Blue = with tannins. Log (CFU): log-transformed colony forming units.

The Table 3, summarizes the effect of tannin supplementation on the growth of *Faecalibacterium*, expressed as Log CFU, across nine individual gut microbiomes (S1 to S9) and as a general mean. The treatments include combinations of different substrates with and without tannins. Results are presented as means ± standard error, and statistical significance is indicated using letter groupings.

As shown in Table 3, Duncan’s groupings indicate significant differences among treatments within each microbiome. A clear pattern emerges in the whole grain substrates (K55, K55AE, Waxy), where the tannin-supplemented treatments frequently fall into higher statistical groups compared to their non-tannin counterparts. This tendency is particularly evident in microbiomes S1, S5, S7, S8, and S9, while in others, such as S2 and S4, the two conditions share similar groupings, suggesting no significant difference.

For purify substrates (High Amylose, Potato, Corn Starch), the response to tannins is less consistent. In several microbiomes (S1, S2, S5, S6, S8), tannin and non-tannin treatments belong to the same statistical group, indicating similar growth. However, in certain microbiomes (notably S3 and S7 with Potato starch) the non-tannin treatments occupy higher groups than the tannin supplemented ones, suggesting localized differences.

Across all microbiomes, the highest values are frequently observed in High Amylose with tannins, especially in microbiomes like S3 and S5, where this treatment ranks at the top grouping. Conversely, K55 without tannins shows the lowest values, particularly in S1 and S9, remaining statistically separated from most other treatments.

The control treatment, which lacks added substrate, generally occupies intermediate groupings. It often aligns with whole grain treatments without tannins but remains below most starch-based treatments across microbiomes. Overall, ANOVA confirmed significant differences among treatment ($p < 0.0001$), and coefficients of variation ranging from 2.18% to 10.68% indicate consistent reproducibility across microb

Table 33

Effect of Tannin Supplementation on Faecalibacterium Growth (log CFU) Across Nine Individual Gut

	Tannins	**Substrate**	**Microbiome S1**	**Microbiome S2**	**Microbiome S3**	**Microbiome S4**	**Microbiome S5**	**Microbiome S6**	**Microbiome S7**	**Microbiome S8**	**Microbiome S9**
			LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE	LOG UFC Mean ± SE
Treatments	Yes	K55	2.82 ± 0.27 ^{bcd}	4.24 ± 0.08 ^{cd}	4.63 ± 0.04 ^{abc}	2.80 ± 0.08 ^{cd}	3.05 ± 0.12 ^{ab}	3.20 ± 0.08 ^{a}	3.93 ± 0.05 ^{c}	3.88 ± 0.04 ^{c}	3.38 ± 0.03 ^{d}
		K55AE	2.67 ± 0.06 ^{de}	4.19 ± 0.09 ^{cd}	4.52 ± 0.05 ^{bcd}	2.84 ± 0.10 ^{cd}	2.89 ± 0.05 ^{bcd}	3.21 ± 0.09 ^{a}	4.00 ± 0.09 ^{c}	4.00 ± 0.07 ^{b}	3.59 ± 0.02 ^{cd}
		Waxy	2.80 ± 0.10 ^{cde}	4.24 ± 0.04 ^{cd}	4.71 ± 0.06 ^{ab}	2.93 ± 0.04 ^{cd}	2.86 ± 0.07 ^{bcd}	3.32 ± 0.09 ^{a}	4.00 ± 0.09 ^{c}	3.76 ± 0.05 ^{d}	3.45 ± 0.02 ^{cd}
		Potato Starch	2.95 ± 0.15 ^{bc}	4.03 ± 0.06 ^{cde}	4.28 ± 0.03 ^{e}	3.19 ± 0.04 ^{bc}	2.89 ± 0.05 ^{bc}	2.65 ± 0.08 ^{a}	4.37 ± 0.07 ^{d}	3.70 ± 0.07 ^{de}	3.50 ± 0.02 ^{cd}
		Corn Starch	2.90 ± 0.06 ^{bcd}	3.97 ± 0.06 ^{cde}	4.33 ± 0.07 ^{de}	3.06 ± 0.05 ^{bc}	3.37 ± 0.07 ^{a}	2.72 ± 0.04 ^{a}	4.15 ± 0.06 ^{b}	3.67 ± 0.07 ^{e}	3.41 ± 0.05 ^{cd}
		High Amylose	3.09 ± 0.11 ^{ab}	4.30 ± 0.14 ^{cd}	4.41 ± 0.05 ^{de}	3.20 ± 0.06 ^{bc}	3.12 ± 0.08 ^{ab}	3.18 ± 0.04 ^{a}	4.54 ± 0.03 ^{a}	3.71 ± 0.09 ^{de}	3.62 ± 0.02 ^{cd}
	NO	K55	2.27 ± 0.15 ^{f}	3.96 ± 0.09 ^{cde}	4.32 ± 0.06 ^{de}	2.31 ± 0.11 ^{e}	2.16 ± 0.11 ^{g}	2.88 ± 0.04 ^{a}	3.21 ± 0.02 ^{e}	3.46 ± 0.04 ^{f}	3.09 ± 0.05 ^{e}
		K55AE	2.07 ± 0.30 ^{f}	3.74 ± 0.07 ^{e}	4.26 ± 0.07 ^{e}	2.33 ± 0.05 ^{e}	2.48 ± 0.06 ^{defg}	2.98 ± 0.07 ^{a}	3.84 ± 0.07 ^{c}	3.44 ± 0.03 ^{f}	2.91 ± 0.10 ^{e}
		Waxy	2.17 ± 0.27 ^{f}	4.05 ± 0.04 ^{cde}	4.28 ± 0.05 ^{e}	2.56 ± 0.11 ^{de}	2.19 ± 0.11 ^{fg}	3.08 ± 0.13 ^{a}	3.48 ± 0.05 ^{e}	3.53 ± 0.04 ^{f}	2.89 ± 0.05 ^{e}
		Potato Starch	2.80 ± 0.27 ^{cde}	5.02 ± 0.07 ^{a}	4.39 ± 0.05 ^{de}	4.09 ± 0.09 ^{a}	2.47 ± 0.08 ^{defg}	3.02 ± 0.07 ^{a}	3.96 ± 0.06 ^{c}	4.06 ± 0.06 ^{ab}	3.95 ± 0.07 ^{a}
		Corn Starch	2.54 ± 0.28 ^{e}	4.76 ± 0.05 ^{ab}	4.53 ± 0.09 ^{bcd}	3.95 ± 0.03 ^{a}	2.48 ± 0.06 ^{cdef}	2.96 ± 0.30 ^{a}	3.81 ± 0.03 ^{c}	3.75 ± 0.04 ^{d}	3.95 ± 0.04 ^{a}
		High Amylose	2.60 ± 0.23 ^{e}	4.70 ± 0.03 ^{ab}	4.75 ± 0.08 ^{a}	3.87 ± 0.03 ^{a}	2.29 ± 0.03 ^{efe}	2.87 ± 0.04 ^{a}	3.99 ± 0.05 ^{d}	4.11 ± 0.06 ^{a}	3.91 ± 0.11 ^{ab}
Control_No			3.22 ± 0.07 ^{a}	4.15 ± 0.03 ^{cd}	4.33 ± 0.06 ^{de}	3.06 ± 0.08 ^{bc}	2.70 ± 0.07 ^{bcde}	2.95 ± 0.30 ^{a}	3.93 ± 0.04 ^{c}	3.63 ± 0.06 ^{e}	3.12 ± 0.05 ^{e}
Pr > F			< 0.0001	<0.0001	< 0.0001	< 0.0001	< 0.0001	0.7708	< 0.0001	< 0.0001	< 0.0001
CV (%)			6.37%	6.91%	3.64%	9.29%	6.96%	10.68%	3.60%	2.18%	5.97%

Not:. Tannins: presence (Yes) or absence (No) of tannins in the substrate. Control_No: fermentation control without tannins. SE: standard error. CV: coefficient of variation. Pr > F: probability value from ANOVA. Letters a-f indicate statistically significant differences between treatments within each column, according to Duncan's multiple comparison test (α = 0.05).

The contrasting responses observed between whole grain and starch-based substrates suggest that tannin activity is strongly influenced by the food matrix. In whole grain treatments (K55, K55AE, Waxy), tannin supplemented conditions were generally placed in higher Duncan groupings thantheir non-tannin counterparts, indicating a positive shift in *Faecal ibacterium* abundance. In contrast, starch-based treatments (High Amylose, Potato, Corn Starch) showed minimal or inconsistent differences between tannin and non-tannin conditions, with several microbiomes sharing the different statistical groupings and occasional cases non-tannin treatments reached higher means values.

These patterns can be explained by the physicochemical interactions of tannins with food matrix components. Condensed tannins bind strongly to seed proteins (prolamins, glutelins) and dietary fibers via hydrogen bonding and hydrophobic interactions, reducing their bioavailability and moderating their antimicrobial activity (Hagerman & Butler, 1980; Ozdal et al., 2016; Tuohy et al., 2012). Whole grain matrices contain these binding components, creating a buffered environment where tannins exert less direct antimicrobial pressure. In contrast, purified starch substrates lack proteins and fibers, leaving tannins chemically free and potentially more bioactive, which may explain the absence of significant shifts in *Faecalibacterium* abundance in these matrices.

The metabolic features of *Faecalibacterium* further support this interpretation. This genus is a key butyrate producer that relies on external acetate to fuel butyryl-CoA:acetate CoA-transferase pathways, as it cannot synthesize acetate de novo (Duncan et al., 2002). Moreover, *Faecalibacterium* exhibits limited enzymatic capacity to degrade complex polysaccharides such as starch, xylan, or arabinogalactan, depending instead on simple sugars or oligosaccharides liberated by other fermenters (Lopez-Siles et al., 2017). In whole grain matrices, the presence of fibers fosters cross-feeding interactions, other microbes break down fiber, releasing acetate and oligosaccharides that *Faecalibacterium* can utilize (Miquel et al., 2014). When tannins bind to these grain components, their antimicrobial activity is moderated and competing taxa may be more strongly affected, indirectly favoring *Faecalibacterium* growth.

Evidence from previous studies supports this interpretation. Yang et al. (2022) reported that *Faecalibacterium* not only tolerates condensed tannins but can increase its abundance in their presence, particularly quebracho derived tannins, with increases up to 1 log CFU in pure cultures and in vitro fermentations. Similarly, Tuohy et al. (2012) demonstrated that plant polyphenols can selectively suppress pathogenic taxa such as *Clostridium perfringens* and *Clostridium difficile* while having limited effects on beneficial commensals, potentially reducing microbial competition. Collectively, these findings align with the patterns observed, tannins in whole grain matrices are linked to higher *Faecalibacterium* counts, while starch matrices remain exhibiting inconsistent responses

Conclusions

Tannin supplementation stimulated *Faecalibacterium* growth in whole grain matrices (K55, K55AE, Waxy Corn) but not in purified starches (High Amylose, Potato, Corn Starch), where values tended to be slightly lower. This suggests that the effect of tannins depends on the complexity of the food matrix, likely due to interactions with proteins and fibers that reduce their antimicrobial activity.

Tannins significantly decreased butyrate and acetate concentrations. In contrast, propionate levels remained relatively unaffected, indicating that microbial pathways for propionate synthesis may be less sensitive to tannin exposure.

High Amylose starches promoted higher butyrate production and *Faecalibacterium* growth in the absence of tannins, whereas whole grain matrices stimulated *Faecalibacterium* only under tannin-supplemented conditions. This highlights how substrate structure influences both SCFA profiles and microbial responses to tannins.

Recommendations

Future studies should include 16S rRNA gene sequencing or metagenomic analysis to characterize the full microbial community composition present in each individual microbiome. This would allow a better understanding of which microbial taxa are responsible for the observed variations in SCFA production and Faecalibacterium growth under different treatments.

It is recommended to explore the metabolic pathways activated in response to tannin supplementation, particularly in whole grain matrices, to determine whether tannins act directly as co-substrates or indirectly by modulating microbial competition.

Given the selective effect of tannins across different matrices, future research should also evaluate different concentrations and types of polyphenols, including hydrolysable tannins and their interaction with other dietary components, to better define optimal formulations for microbiome targeted functional foods.

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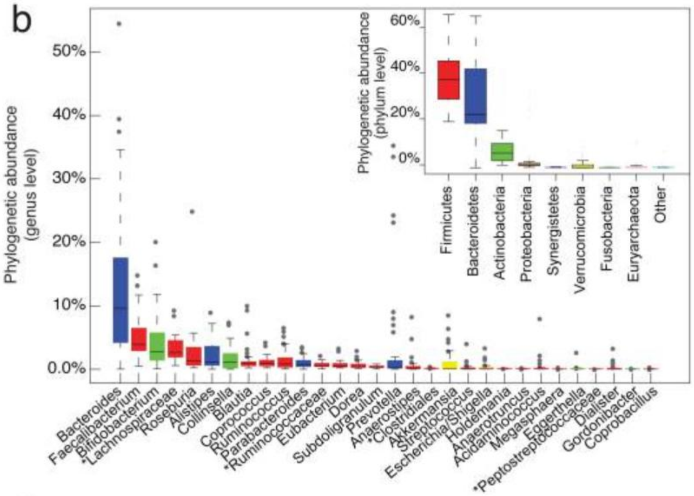
Appendices

Appendice A

Functional and phylogenetic profiles of human gut microbiome.



Note: Source: Reprinted from *Enterotypes of the human gut microbiome*, by Arumugam et al., 2011, *Nature*, 473(7346), p. 174–180.

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