Total polyphenols and antioxidant capacity in kombucha from different teas and their effects on intestinal health in-vitro intestinal epithelial tissue model

Rucha Rane

Company Supervisor – Nicholas Rosenstock
University Supervisor – Olena Prykhodko
University Examiner – Claudia Lazarte

KLTM02
Department of Process and Life Science,
Faculty of Engineering, LTH
Spring, 2024

PREFACE

As we explore the new horizons of functional foods, this report brings together various health benefits of a popular fermented beverage, kombucha. This topic was chosen to highlight the importance of fermentation on functional foods and the associated nutritional benefits in humans. I am extremely contended with the new techniques, methodologies, and skills I have learnt during this journey and am super proud of myself.

This project wouldn't have been possible without the help of many people, most importantly Olena Prykhodko, who has always helped and guided me for all minute details in this project and has participated in it with utmost interest and experience. I would also like to thank Nicholas Rosenstock for sharing with me wonderful facts about kombucha and assisting me in its preparation and sampling. Thanks for being so curious about the science and the results obtained in this project.

I am grateful to Claudia Lazarte for her thorough feedback and evaluation of this project. Her input and positive critique helped me in improving this thesis report. I would like to extend my gratitude towards Olexander Fedvik for always assisting me with all the doubts I had and arranging all the apparatus and chemicals in this project.

Lastly, thanks to the entire staff at Roots of Malmö, my fellow thesis mates at the department for constant moral support, my parents (Rajesh Rane and Purva Rane), and my friends in Lund (Aditya, Manoj, Aarushi, Anshul, Lola, and Eva) and in India (Saurav, Rinkal, Karan and Jitesh) for being there with endless support.

ABSTRACT

Kombucha contains a plethora of compounds that contribute to its health benefits apart from its probiotic effects. This project was conducted to determine the total polyphenolic content (TPC) and total antioxidant content (TAC) of kombucha prepared with black tea, oolong tea, and green tea as a function of fermentation time (day 0 to day 18). Amongst all the kombucha samples, green tea kombucha on day 9 (GTK 9) had the highest TPC (506.97 ± 22.28 µg GAE/mL) and TAC (440.93 ± 1.46 µmoles TE/ mL) and it was chosen to evaluate its effect on the transepithelial electrical resistance (TEER), antioxidative capacity (AOC), tissue permeability and cell viability using a 3D model of the intestinal epithelial tissues. For these analyses, two different concentrations of gallic acid (GA), 500 and 2000 µg/mL were also used since GA, is a simple phenolic compound that is also one of the end-products of polyphenol fermentation and digestion and has known benefits on the intestinal epithelial health. All the results were compared to a negative control (NC), treated with PBS. GTK 9 had a similar effect as 500 µg/ml GA for TEER, where their effect wasn't significantly different than the NC, and for AOC, where their effect significantly increased the AOC of the tissues. GTK 9 increased the tissue permeability significantly more than the NC, but this increase was not above the threshold level to be harmful to the tissues. All the treatments proved to increase cell viability, making them cytoprotective. Overall, both the concentrations of GA and GTK 9 proved to be beneficial for improving the health of the intestinal epithelial tissues.

Keywords – Kombucha, Total polyphenolic content, Total antioxidant capacity, Intestinal epithelial tissue model, Gallic acid

TABLE OF CONTENTS

ABBREVIATION

500 GA -	500 ug/mL gallic acid
2000 GA -	2000 ug/mL gallic acid
AOC -	Antioxidative capacity
BTK -	Black tea kombucha
GA -	Gallic acid
GTK -	Green tea kombucha
GTK 9 –	Green tea kombucha on day 9
LY -	Lucifer yellow
OTK -	Oolong tea kombucha
PBS -	Phosphate buffered saline
PBS (+ Mg, Ca) -	Phosphate buffered saline with magnesium and calcium ions
PBS (- Mg, Ca) -	Phosphate buffered saline without magnesium and calcium ions PBS
PFA -	Paraformaldehyde
RH -	Relative humidity
RT -	Room temperature
TAC -	Total antioxidative capacity
TEER -	Transepithelial electrical resistance
TPC -	Total polyphenolic content
TSS -	Total soluble solids

1. INTRODUCTION

1.1. Kombucha

Functional foods and beverages have gained a lot of popularity and consumer attention in the past few years because of their additional health benefits on physical and mental health beyond basic nutrition. One such functional beverage that has received a lot of focus worldwide is kombucha (1).

Originally processed in China, kombucha is a famous fermented tea drink that is non-alcoholic [<0.5% (v/v)] and has been widely consumed all over the world, especially in Japan, Russia, and Eastern Europe since World War II (1-3). Kombucha is a sweet-sour and carbonated beverage that is prepared by fermenting tea and sugar mixture under aerobic conditions by a symbiotic system of SCOBY (Symbiotic Colony of Bacteria and Yeast) (1,4,5). After the fermentation, the sour liquid product is obtained containing a floating bio-cellulose pellicle (6).

The most common microorganisms found in kombucha include acetic acid bacteria like *Acetobacter*, *Gluconacetobacter*, and *Komagataibacter* and some lactic acid bacteria like *Lactobacillus bulgaricus* along with different yeast genera like *Saccharomyces*, *Dekkera*, *Zygosaccharomyces* and *Pichia* (3,4). During the fermentation of kombucha, yeast initially converts sugar into ethanol. Afterward, bacteria further converts ethanol into various organic acids, including acetic acid, glucuronic acid, and gluconic acid. (3,4,7).

The functional characteristics of kombucha may vary depending on the type of tea used, which contains a range of constituents such as phenolic compounds, caffeine, theaflavins, theanine, organic acids, micronutrients, and cell metabolites produced during tea production (7). Thus, the tea type used to prepare kombucha affects its chemical composition and bioactive components.

Black tea, green tea, and oolong tea are all derived from the same plant species, *Camellia sinensis* but they are produced by different processing methods leading to specific flavour and aroma profiles. Black tea undergoes complete enzymatic oxidation or fermentation during processing whereas green and oolong tea undergo minimal and partial enzymatic oxidation respectively (8). The difference in enzymatic oxidation time leads to a difference in the polyphenolic content of the tea, which is responsible for various health benefits in tea (7).

1.2. Polyphenols

Polyphenols are secondary metabolites known to improve human well-being (9). Polyphenolic compounds are further divided into two groups- flavonoids and non-flavonoids, each group containing various compounds like anthocyanins, phenolic acids, flavanols, kaempferol, etc

(10). The intake of phenolic substances can lower the risk or even postpone the onset of illnesses such as cancer, metabolic syndrome, and neurological diseases by altering signalling pathways including inflammation, oxidative damage, and apoptosis (10).

The antioxidant activity of polyphenols is one of their most important biological functions. These compounds have a direct potential for scavenging reactive oxygen species due to the presence of one or more hydroxyl functional groups (OH^-) attached to a carbon aromatic ring in their chemical structure. Furthermore, they have the potential to indirectly enhance the activity of antioxidant enzymes (10). The presence of various vitamins like vitamin C, vitamin B2, or vitamin E is known to impart antioxidant effects in humans as well (9).

During fermentation, bacteria increase the properties of food by being anti-pathogenic and anti-microbial. Furthermore, these bacteria have the ability to break down complex phenolic compounds into simple phenolic acids, increasing their antioxidative capacity and making them more accessible to human cells (11).

1.3. Effects of fermented polyphenols on human health

One of the most abundant phenolic compounds found in many medicinal plants and produced by fermentation of complex polyphenols is gallic acid (12,13). Previous studies have shown gallic acid to have several advantages in improving the functionality of intestinal epithelial tissue permeability, strengthening its antioxidative capacity, and having cytoprotective properties (13-15).

The presence of bioactive components in polyphenol-rich beverages such as kombucha imparts various health benefits like antioxidant activities, oxidative stress-reducing capacity, antimicrobial activities, and anti-cancerous properties (4).

The human intestinal tissues have antioxidative potential but in case of oxidative stress, where the production of free radicals and reactive oxygen species is more than the cellular antioxidants, the chances of tissue injuries and the onset of deteriorating diseases like cancer, heart diseases, and inflammatory bowel disease increases (16). In this case, polyphenols and their fermented derivatives can strengthen the antioxidative capacity of the intestinal tissues (17).

It's important to highlight that fermented polyphenols have been found to exert an influence on the integrity of the intestinal epithelial barrier function (13, 18). This effect, in turn, contributes to the enhancement of nutrient uptake, particularly by facilitating the absorption of essential amino acids (13).

1.4. 3D human intestinal tissue model

The 3D human intestinal tissue model is a unique model of reconstructed intestinal tissue from stem cells of healthy donors raised to an air-liquid interface to emulate the real-life scenario. In this project, conducting in-vitro analysis on the integrity and permeability of intestinal cells offers valuable insights into the direct impact of food compounds on a healthy intestine. This analysis aids in enhancing our comprehension of tissue integrity regulation, a pivotal factor in the onset of 'leaky' gut syndrome. This syndrome involves the penetration of harmful substances such as bacterial walls or bacteria themselves into the bloodstream, leading to potential health complications (19).

Cell viability, defined as the percentage of live cells in the total cells is a crucial parameter to quantify for evaluating the effect of a particular treatment on the cells (20). A cytotoxic substance will decrease cell viability, killing the cells whereas a cytoprotective substance has the opposite effect.

The above-mentioned parameters have been mainly evaluated for various substances via ex-vitro models by conducting experiments on rodents because of the physiological similarities and ease of accessing various regions inside the body. However, the data obtained by ex-vivo methods cannot be fully used for human studies because of the differences in organ morphology, gut microbiome, food intake, etc (21). A better solution to elucidate this problem would be to use an in-vitro model that gives a clear picture of what would happen in the gastrointestinal system, is easy to work with, gives rapid results, and is a more ethical practice.

2. AIM AND OBJECTIVES

2.1 AIM

The aim of this study was to determine the total polyphenolic content (TPC), and total antioxidative capacity (TAC) of kombucha made with black tea, green tea, and oolong tea using the company recipe of Roots of Malmö. In addition, to evaluate the effect of gallic acid (GA), a simple phenolic acid, and the kombucha itself to see its effect on intestinal permeability, antioxidative capacity, and cell viability.

2.2 OBJECTIVES

1. To carry out fermentation of black tea kombucha (BTK), oolong tea kombucha (OTK), and green tea kombucha (GTK) at Roots of Malmö.
2. To monitor the pH and total soluble solids (TSS) as ºbrix of all the kombucha from Day 0 to Day 18.
3. To determine the TPC of all the kombucha samples by the Folin-Ciocalteu method and evaluate which samples are to be tested for further analysis at the PLE department, LTH.
4. To evaluate the TAC of the selected kombucha samples by 2 methods, DPPH and ABTS at the PLE department, LTH.
5. To expose the intestinal epithelial tissues using a 3D in-vitro model, SMI-100 to two concentrations (500 ug/mL and 2000 ug/mL) of GA and the best kombucha sample and to evaluate their transepithelial electrical resistance (TEER) and intestinal permeability, antioxidative capacity (AOC) and cell viability at the PLE department, LTH.

3. MATERIALS AND METHODS

3.1 Preparation of kombucha

Three different types of kombuchas were prepared using 3 different tea leaves, namely black tea, oolong tea, and green tea. All the ingredients required to make kombucha (the tea leaves, sucrose, and the starter culture) were obtained from the company, Roots of Malmö, Sweden where the kombucha was prepared in sterile conditions and allowed to ferment.

Figure 1 consists of a flowchart, briefly explaining the steps undertaken to prepare kombucha at Roots of Malmö. The first step in kombucha preparation was to brew tea. Around 6.5L of water was boiled in a 10 L pot. After boiling, 60 g of tea was added to it. This mixture was allowed to simmer at a low temp for about 7 minutes after which 720 g of sucrose was added to the pot and the tea was allowed to boil for another 3 minutes. At the end of the second boil, the stove was turned off and the tea was allowed to steep for 5 minutes.

Then the tea was strained and cooled in a water bath until the temperature reached below 40 °C. It was transferred to a bucket (with measurement markings) and cold water was added to make the volume 11.3L. 200 ml was taken for measurements and 900 mL of starter culture (old kombucha broth that was continuously mixed to ensure a homogenous mixture) was added to the bucket to make the total volume 12 L. 2.5 L of this prepared kombucha was then transferred to 4 glass jars of 3L capacity to obtain 4 fermentation replicates for each tea type.

The same procedure was repeated for all the types of tea leaves. All the jars were then covered with a cotton cloth, sealed with rubber bands, and kept at room temperature (RT) of 21 °C to let them ferment for a maximum of 18 days.

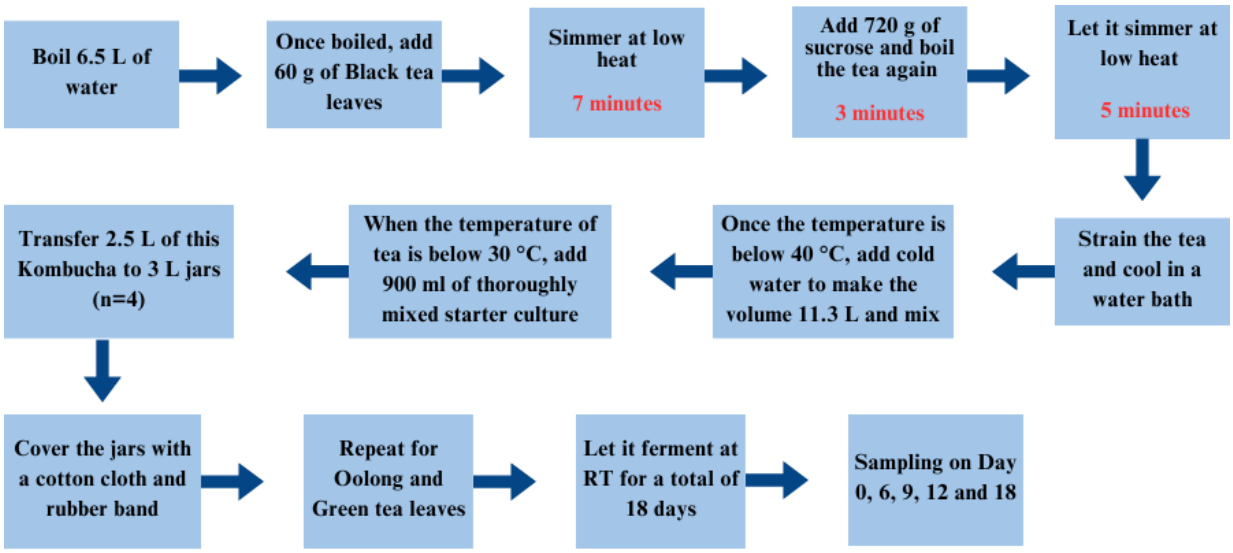

Figure 1 – Flowchart describing the method to prepare kombucha.

3.2 Sampling

Sampling was done on days 0, 6, 9, 12, and 18 by carefully taking the kombucha from the middle of the jar (without disturbing the pellicle formed on the top) using a syringe and transferring it to polypropylene (PPE) 50 ml tubes. All the samples were taken in 4 aliquots and stored at -18 ºC at Roots of Malmö until further analysis. At the end of the fermentation, the pellicle formed on the top was weighed and stored in the freezer.

3.3 Measuring pH and total soluble solids (TSS) as °Brix

pH and TSS as °Brix were measured for the few samples at Roots of Malmö and for all the samples at LTH. pH was measured using MeterLab PHM 210 instrument, calibrated using pH 4 and 7 provided by the company and °Brix of the samples was measured directly using a refractometer (ABBE, Optic ivymenSystem) at the PLE department, LTH. All the measurements were taken in duplicates and water was used as a blank for measuring TSS.

3.4 Determining the total polyphenolic content (TPC)

The TPC was determined by the Folin-Ciocalteu method described in the method published by Sutthiphatkul et al. (2022) (4), with some modifications. Briefly, 20 µL of kombucha samples were mixed with 100 µL of 10% (v/v) Folin-Ciocalteu reagent (Sigma-Aldrich). After 5 minutes, 80 µL of 7.5% (w/v) sodium carbonate (Sigma-Aldrich) was added and the mixture was incubated in the dark for 30 minutes. The absorbance of all the samples was measured using a SPECTROstar Nano Microplate reader by BMG Labtech at 765 nm by taking distilled water (DW) as blank.

For the calibration curve, GA (Sigma-Aldrich) solutions were prepared with DW in the concentration range of 100-500 µg/mL. The TPC content of the kombucha samples was calculated using the following equation ($y = 0.0083x + 0.1653$) obtained from the GA calibration curve and the results were expressed as µg GA equivalent per mL of kombucha sample (µg GAE/mL).

3.5 Determining the total antioxidant Capacity (TAC)

The TAC was determined using two methods, ABTS and DPPH, as both the methods have similar working principles but different affinities towards polar or non-polar groups, and are suitable depending on the type of sample.

3.5.1. DPPH assay

DPPH method was performed by first preparing 100 mL of 0.2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich) solution in methanol (Sigma-Aldrich, purity ≥ 99.6%). The assay was carried out by procedures published by Sutthiphatkul et al. (2022) (4) with some modifications. In brief, 250 µL of kombucha samples were mixed with 750 µL of 0.2 mM DPPH solution and incubated for 30 min in the dark at RT. After the end of incubation time, 150 µL of each sample mixture was pipetted into a 96-well plate and the absorbance of the samples was measured at 517 nm using SPECTROstar Nano Microplate reader by BMG Labtech by considering DW as the blank.

For the calibration curve, 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox) (Sigma-Aldrich) solutions were prepared with DW in the concentration range of 200-1000 µmoles /mL. The TAC (DPPH) content of the kombucha samples was calculated using the following equation (y = 0.0006x - 0.0045) obtained from the trolox calibration curve and the results were expressed as µ Moles trolox equivalent per mL of kombucha sample (µmoles TE /mL).

3.5.2. ABTS assay

The ABTS method was performed by first preparing the ABTS radical solution. For this, an equal proportion of 7 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich) was mixed with 2.45 mM Potassium persulfate and incubated in dark at RT for around 16 hours. In a nutshell, the ABTS assay was carried out by procedures published by Sutthiphatkul et al. (2022) (4) with some modifications. 250 µL of kombucha samples were mixed with 750 µL of ABTS radical solution and were incubated for 30 min in the dark at RT. After the end of incubation time, 150 µL of each sample mixture was pipetted into a 96-well plate and the absorbance of the samples was measured at 734 nm using SPECTROstar Nano Microplate reader by BMG Labtech by considering DW as the blank.

For the calibration curve, trolox solutions were prepared with DW in the concentration range of 200-1000 µmoles /mL. The TAC (ABTS) content of the kombucha samples was calculated using the following equation (y = -0.0207x + 8.9759) obtained from the trolox calibration curve and the results were expressed as µmoles trolox equivalent per mL of kombucha sample (µmoles TE/mL).

3.6 Intestinal epithelial tissue model (SMI-100)

Intestinal epithelial tissues (SMI-100), n=24 were received from MatTek In Vitro Life Science Laboratories, Bratislava, Slovakia. The tissues were received on feeding media gel and were pre-equilibrated overnight, before conducting experiments according to Kostiuchenko et al. (2022) (22) and followed by protocol provided by MatTek Corporation, 2023 (23). For this,

under sterile conditions, 5 mL of preheated SMI-100 MM medium (MatTek) was pipetted in 12 well-bottom plates (n=2). Next, the tissue inserts were gently removed from the gel and transferred to the hanging top 12 plate which was then put on top of the bottom plate. 100 µL of SMI-100-MM was pipette into the apical side of the tissue and the plates were then covered with the lid. The entire assembled plate was incubated overnight at 37 ºC, 5% CO_2, and 95% relative humidity (RH).

3.7 Tissue experimental design

After re-equilibration, the next morning the tissues were taken out of the incubator under sterile conditions. The apical fluid was aspirated and expelled from the tissue inserts and the tissues were exposed to 100 µL of different treatments as described in Figure 2. Sterilized Dulbecco's phosphate buffered saline with magnesium and calcium ions [PBS (+ Mg, Ca)] (n=6) (Gibco, Thermo Fischer Scientific), filter sterilized 500 µg/mL of GA (500 GA) prepared in PBS (+ Mg, Ca) (n=6), 2000 µg/mL of GA (2000 GA) prepared in PBS (+ Mg, Ca) (n=6) and sterilized (at 121ºC) green tea kombucha of day 9 (GTK 9) (n=6). The tissues were exposed to corresponding treatments for 24 hours at +37 ºC, 5% CO_2, and 95% RH.

As shown in Figure 2, for determining the antioxidative capacity, 8 tissue inserts were used, 2 from each treatment group with PBS (+Mg, Ca) being the negative control. To determine the intestinal permeability by lucifer yellow (LY) passage, 8 tissue inserts, 2 of each group were used. For the MTT assay, 10 tissue inserts were used, 2 of each group and the 2 tissues that were used as a negative control for the permeability test (4 tissue inserts that were PBS (+Mg, Ca) treated were used as controls for MTT assay).

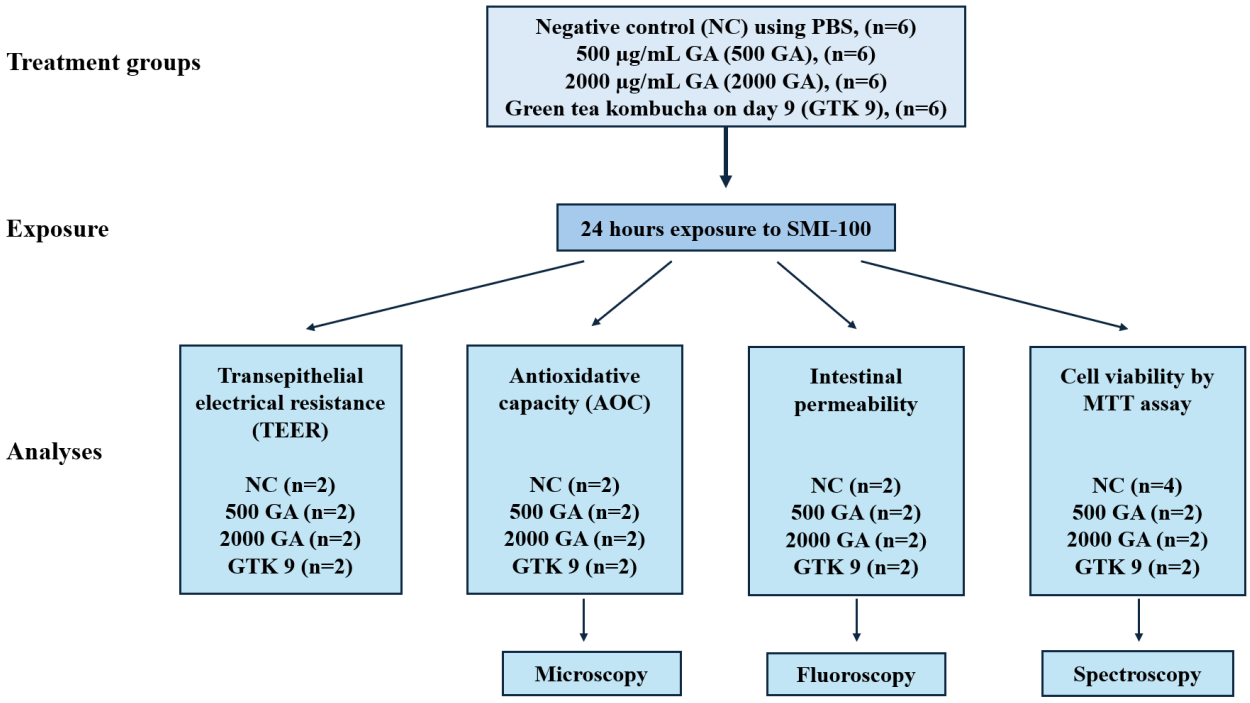

Figure 2 – Flowchart describing the experimental plan for SMI-100 model.

3.8 Transepithelial electrical resistance (TEER) measurement

TEER was measured for the tissue inserts after 24 hrs of treatment exposure using EVOM2 Epithelial Voltohmmeter by World Precision Industries (WPI) to take the readings with EndOhm tissue resistance measurements chamber. After the completion of treatment, the apical fluid for all the inserts was expelled and they were taken out of the feeding media plate to be washed in magnesium and calcium-free sterile PBS [PBS (-Mg, Ca)] (Gibco, Thermo Fischer Scientific) for a least of 3 times.

After that, eight of the inserts (2 from each treatment) were washed in sterilized 100 mM potassium chloride (KCl) (Sigma-Aldrich) and put in a 24-well plate with 500 µL of KCl as the basal fluid and 400 µL as the apical fluid (23). The electrode chamber was filled with 2 mL of KCl, and the resistance of tissues was measured by keeping each tissue insert in the chamber and considering the empty insert (with the treated membrane, as provided with EVOM2 by WPI) as a blank.

TEER was calculated using equations 1 and 2 (24),

$R_{tissue} = R_{sample} - R_{blank}$ Equation 1

$TEER_{tissue} = R_{tissue} \times Area_{tissue}$ Equation 2

Where R stands for resistance (Ω) and the $Area_{tissue}$ is 0.6 cm^2 (23)

3.9 Antioxidative capacity (AOC)

To perform the AOC assay, eight tissue inserts, 2 from each treatment group were used. The apical fluid was expelled from the tissue inserts and they were washed with sterilized PBS (-Mg, Ca). 100 µl of 0.5 mM filter sterilized hydrogen peroxide (H_2O_2) was applied to the tissues from the apical side to induce oxidative stress for 1 hour (16). After the completion of 1 hour, the apical fluid was removed and 5 µL of a probe, CellROX Deep Red Reagent (Life Technologies Corporation) was applied on the apical side of the tissue inserts for 30 minutes at +37 ºC, 5% CO_2, and 95% RH. At the endpoint, the tissue inserts were put in tubes containing 4% paraformaldehyde (PFA) (Histolab) and stored overnight at +4ºC for fixation.

The next day, the tissue inserts were removed from the 4% PFA tubes, transferred to tubes containing 0.01 M PBS with 0.02% sodium azide (Sigma-Aldrich), and stored in the dark at refrigerated conditions until microscopy. For analysis of the developed red fluorescence, the tissue inserts were observed under a microscope Olympus connected to a DP74 camera, using a corresponding fluorescent filter and 4x magnification objective. Images were taken at different locations of the tissue insert with an exposure time of 1s by using Imaging Software cellSens Entry (Olympus Life Science). Next, ImageJ2, a Java-based image processing program, provided by the National Institutes of Health (NIH) was used to compare the mean

intensity density of red colour seen in the different tissues and compared to the NC, whose mean intensity density was considered to be 100%.

3.9 Permeability assay

Different buffers were used for permeability assay, and they were prepared by mixing 1 L of 1 x Hank's Balanced Salt Solution (HBSS) (Gibco, Thermo Fischer Scientific) with 10 mL of 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (Gibco, Thermo Fischer Scientific) and adding 1.98 g of glucose (Gibco, Thermo Fischer Scientific). pH of this solution was adjusted to 6.5 for the apical buffer to mimic the intestine's lumen side and to 7.4 for the basolateral buffer to mimic the intestine's blood side. pH adjustments were done using sterilized 1 M hydrochloric acid (Sigma-Aldrich) and 1 M sodium hydroxide (Sigma-Aldrich). Both the buffer solutions were sterilized using a 0.2 µm filter. 100 µM of LY (Invitrogen, Thermo Fisher Scientific) was prepared using the apical side HBSS and sterilized using a 0.2 µm filter while being protected from light.

Prior to the permeability test, eight tissue inserts (2 from each treatment) were washed with sterile PBS (-Mg, Ca) and then transferred to a 24-well plate containing 500 µL of the basolateral fluid (prepared HBSS, pH 7.4) and 100 µL of the apical fluid (prepared HBSS, pH 6.5) containing 100 µM LY was added from the apical side and the plate was kept in the CO_2 incubator for 1 hr. Next, the basal fluids from all the tissue wells were collected and stored in at -18 ºC, protected from light until further analysis. For analysis, the concentration of LY in the basolateral side of the tissues was determined by measuring the fluorescence using Fluoroskan (Thermoscientific) using the filter set (Ex 485 nm, Em 538 nm) (22). The calibration curve was obtained by preparing LY in the apical fluid from the concentration range of 100 µM to 0.001 µM and the concentration of LY was calculated using the equation (y = 2.047x - 0.0611). The results were represented as % uptake of LY by the tissues which was calculated using equations 3 and 4 (23).

$$\% \text{ rejection} = 1 - \left(\frac{Cb}{Ca}\right) \times 100 \qquad \text{Equation 3}$$

$$\% \text{ uptake} = 100 - \% \text{ rejection} \qquad \text{Equation 4}$$

Where, C_b refers to the concentration of LY in the basolateral side and C_a refers to the concentration of LY in the apical side.

3.10 MTT assay

For the MTT assay, 10 washed tissue inserts with sterile PBS (-Mg, Ca) were used, 2 from each treatment group and two to be used as a positive control. As a positive control, tissue inserts were exposed to 100 µL of 0.3% Triton X100 (MatTek) for an hour. Meanwhile, the MTT solution was prepared by mixing the MTT concentrate (MatTek) and the MTT diluent (MatTek) and centrifuging it (300g for 5 minutes, RT). 300 µL of MTT solution was pipetted

on top of the tissues from the apical side in a 24-well plate and the plate was incubated for 3 hours in the CO_2 incubator. After 3 hours, the tissue inserts were thoroughly washed and immersed in a 24-well plate containing 2 mL of the extractant solution for overnight extraction at RT, protected from light. The next day, the extractant was collected from all the inserts' basolateral sides and was analysed by pipetting 200 µL of the sample in triplicates into a 96-well plate and reading the plate's absorbance at 570 nm in a spectrometer using 200 µL of extractant as blank. Cell viability in % was calculated using equation 5 (23),

$$\%viability = 100 \times [OD\ (sample)/\ OD\ (negative\ control)] \qquad \text{Equation 5}$$

Where OD stands for the optical density obtained from the spectrometry.

3.11 Data analysis

The results obtained from all the analyses were expressed as mean ± standard deviation. Statistical analysis in the form of t-test, one-way ANOVA, and two-way ANOVA on different parameters were performed on the obtained results in the GraphPad Prism 10 application.

4. RESULTS AND DISCUSSIONS

4.1 Kombucha preparation and fermentation

Kombucha preparation and fermentation that took place at Roots of Malmö were successful without any visible contamination in the kombucha samples. Figure 3A and 3B shows how the kombuchas looked on day 0 and day 18 respectively.

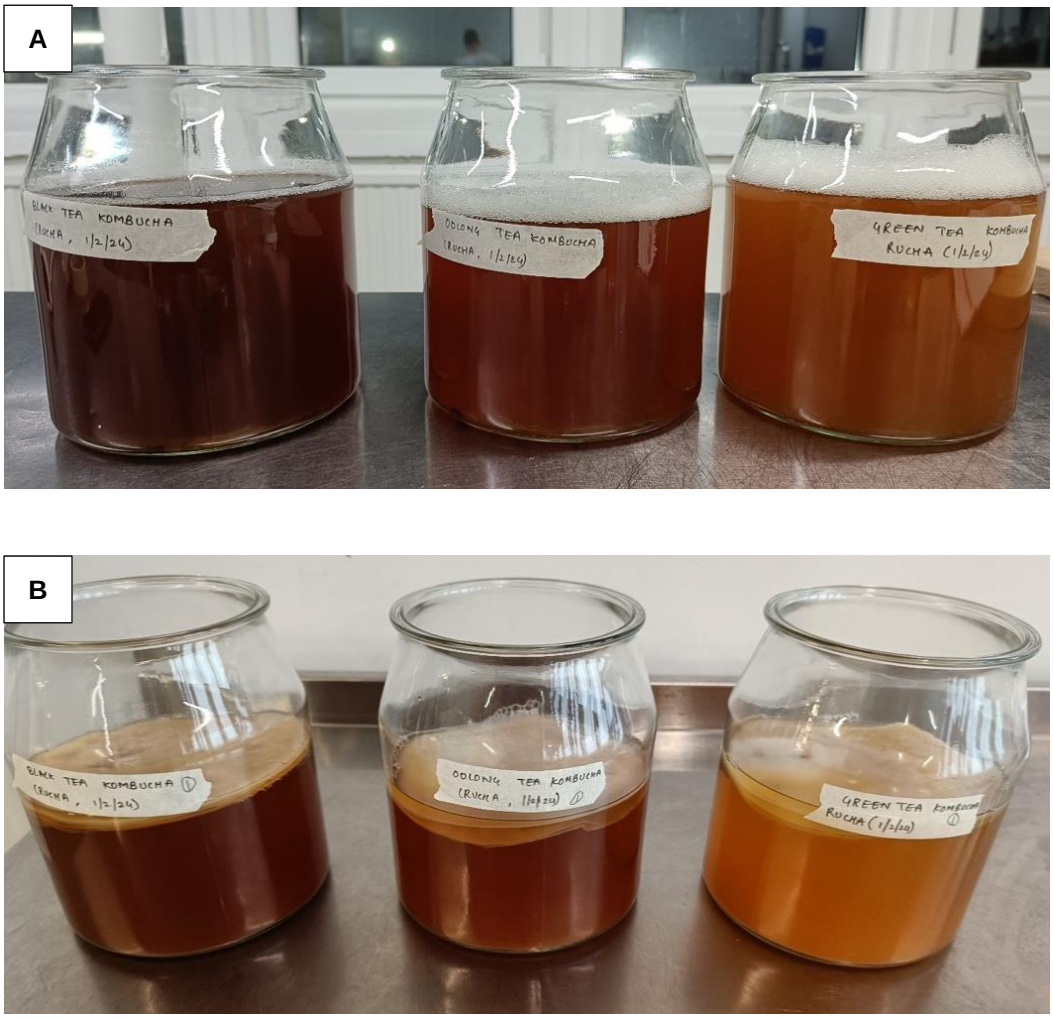

Figure 3 – Kombuchas prepared on (A) day 0 and (B) on day 18
From left to right – Black tea kombucha, oolong tea kombucha, and green tea kombucha.

4.2 pH and total soluble solids (TSS) as °Brix

Figure 4 summarizes the results obtained from pH and TSS analysis of the different kombucha samples at LTH. In the case of all the kombucha samples (BTK, GTK, and OTK), the pH

significantly decreased over the course of fermentation time compared to day 0 ($p < 0.0001$ for BTK and OTK, $p < 0.005$ for GTK). A drop in pH is because of the live microorganisms fermenting the tea solution to make acetic acid and other organic acids. Thus, making the medium more favourable for the microorganisms to grow and proliferate (3,25). The first pH drop from day 0 to day 6 was significantly higher than the consecutive days ($p < 0.005$) for all the kombucha types. From day 6 onwards, GTK had a significantly higher pH than OTK and BTK ($p < 0.05$). However, there was no significant difference ($p > 0.05$) between the pH values among the different kombucha types.

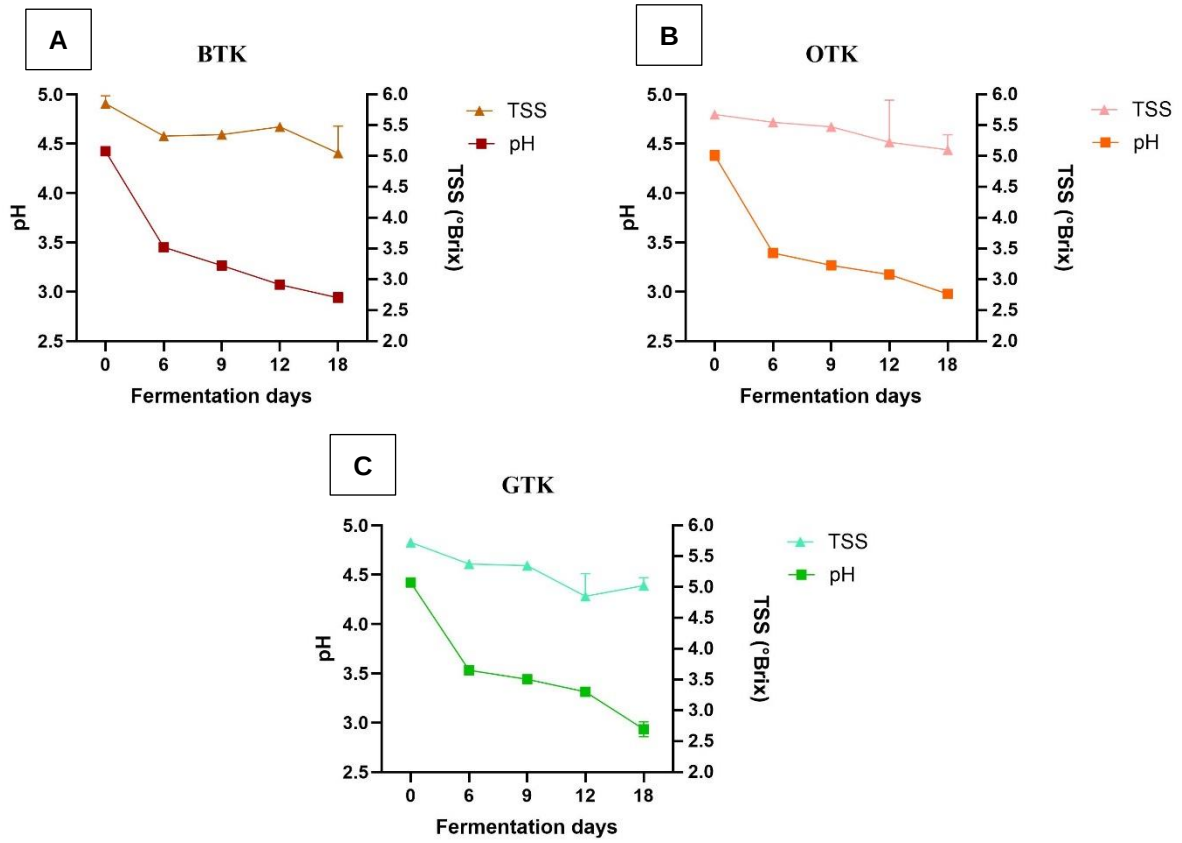

Figure 4 – pH and TSS graph for (A) black tea kombucha, (B) oolong tea kombucha and (C) green tea kombucha as a function of fermentation days.

The total soluble solids, TSS measured as ºBrix decreased significantly ($p < 0.05$) in BTK till day 12 of fermentation, till day 18 for OTK, and for all days except day 12 for GTK compared to day 0. In the case of BTK, the TSS increased on day 12 compared to day 9 but then decreased on day 18. The reason for this is still unclear and needs to be evaluated. For GTK, the TSS dropped significantly ($p < 0.05$) on day 12 compared to day 9 with a larger standard deviation, indicating something went wrong in one of the repetitions. Amongst the kombuchas, OTK had a significantly higher ($p < 0.05$) TSS on day 6 and day 9.

A general trend observed between pH and TSS is that with a decrease in pH, the TSS decreases as well since the micro-organisms are feeding on the sugar to ferment and produce organic acids. The pH of all samples is similar to the study conducted by Suna et al. (2019) (2), where the pH value at the end of fermentation was around 3. The change in TSS quantities is less compared to the research conducted by Wang et al. (2023) (26), where the TSS value after 14

days of fermentation was around 3.25 ºBrix. The reason for this could be the difference in the sugar concentration (50 g/L), starter culture concentration (25 g/L), and fermentation conditions (22 ºC as the fermentation temperature).

4.3 Total polyphenolic content

During fermentation, complex polyphenols might get converted to simpler phenolic compounds like GA, catechins, and epicatechins (5), and the total polyphenolic content is expressed as GA equivalents. Tea is a rich source of complex polyphenols like caffeine, catechins, tannins, etc which get fermented by the microorganisms thus making them more available for human consumption. Black tea contains fewer polyphenols compared to green tea and oolong tea because it is oxidized completely during processing (5).

The starter culture, black tea, oolong tea and green tea used to make kombucha had a TPC (µg GAE/mL) of 341.09 ± 30.51, 357.56 ± 12.84, 316.59 ± 18.42 and 429.34 ± 11.51 respectively (A2). Data obtained from TPC measurement is summarized in Figure 5 in the form of a bar graph. It can be said that the TPC increased till day 12 for BTK and day 9 for OTK and GTK, after which it either decreased or remained constant compared to that time point. This change in TPC content for all the samples was not significantly different ($p > 0.05$) than day 0.

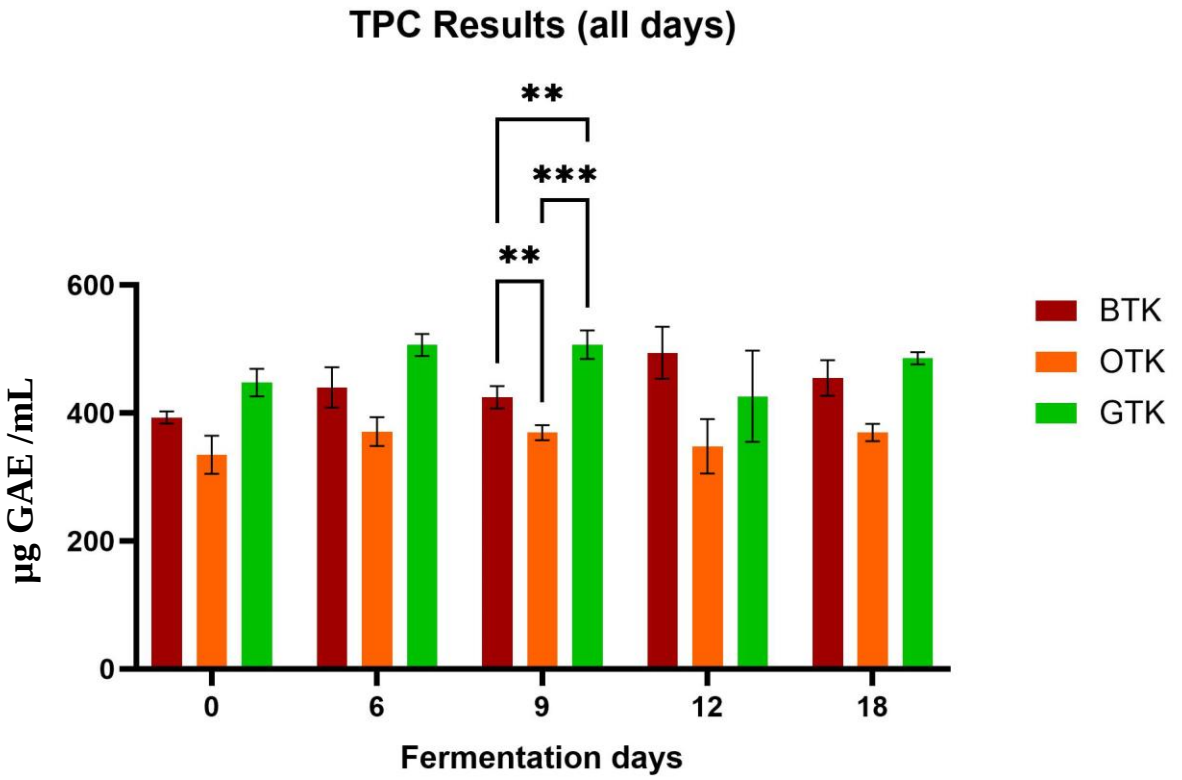

Figure 5 – TPC of black tea kombucha, oolong tea kombucha and green tea kombucha samples as a function of fermentation days (0 to 18) expressed as µg GAE /mL.

GTK had a significantly higher ($p < 0.05$) TPC till day 9 and OTK had a significantly lower ($p < 0.05$) TPC on all days than the others. Samples from day 18 had a lower TPC for all tea types compared to day 9 and thus those samples were not considered for further analysis. Day 9 was the most interesting as all 3 types of kombuchas had significantly different results from each other, and had the least standard deviation and optimum sensory properties.

Fermentation produces many enzymes like cellulase, glucanase, and pectinase that might convert the larger complex polyphenols to smaller phenolic compounds, thus increasing the TPC content (5) but after a certain fermentation time, the TPC decreases or remains constant because of the probability of polyphenol biodegradation (27). A similar trend was found by Değirmencioğlu et al. (2020) (7) where the TPC content of the extractable and hydrolysable fraction of black, green, and oolong tea kombucha decreased after the 8^{th} day of fermentation.

The TPC value obtained for GTK after day 9 of fermentation is very close to the TPC results found in a study conducted by Özyurt (2020) (3) where the value was 472.09 ± 4.94 for GTK at day 9 of fermentation. In the case of BTK, a similar % increase in TPC after day 12 of fermentation was seen as compared to the study conducted by Sutthiphatkul et al. (2022) (4) and Özyurt (2020) (3) where the % increase was around 20-30%. TPC content obtained from this study is specific to the fermentation conditions and composition of the starter culture used.

4.4 Total antioxidant capacity

The TAC of the kombucha samples was measured using 2 assays, DPPH and ABTS. Figure 6 contains the results obtained by the DPPH assay, where the values seem to increase for BTK and OTK with the progress of fermentation, but they appear rather constant for GTK. Amongst the types of kombuchas, GTK has a significantly higher ($p < 0.005$) TAC content than BTK and OTK measured by DPPH. Over the course of fermentation, the TAC value did not change significantly ($p > 0.05$) for all the samples except OTK, where the TAC increased significantly ($p < 0.05$) on day 12 compared to day 0. Day 9 was of interest because of the optimum sensory characteristics of the kombucha samples and the high TAC for GTK.

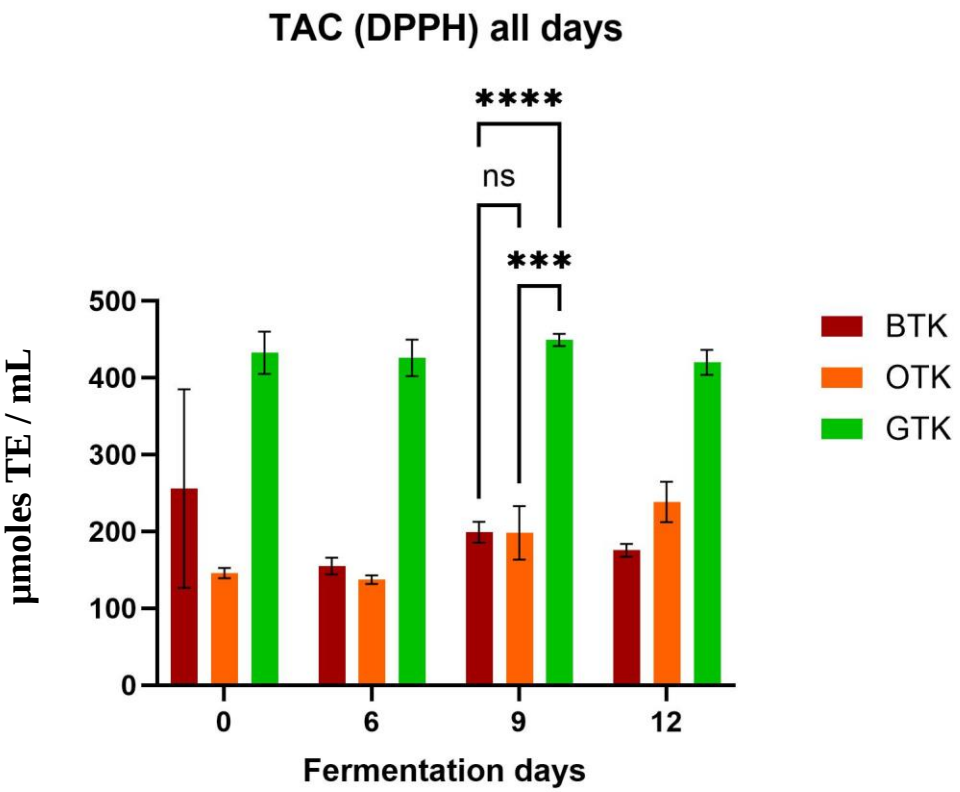

Figure 6 – TAC results by DPPH assay for black tea kombucha, oolong tea kombucha, and green tea kombucha as a function of fermentation days measured as µmoles TE / mL.

Figure 7 summarizes the results obtained by ABTS analysis, where the TAC values seem fairly constant and not significantly different ($p > 0.05$) throughout the fermentation for all the tea types with GTK having the highest TAC compared to others. This finding indicating that GTK has the highest antioxidant capacity, especially with ABTS is similar to the study conducted by Özyurt (2020) (3) and X. Wang et al. (2022) (5), where the fermentation was carried out for 9 days and 15 days respectively. The reason GTK has the highest TAC among other tea types could be because of the difference in processing the tea leaves. Black tea leaves are completely oxidized, destroying the majority of polyphenols and antioxidants but on the other hand, green tea is partially oxidized, and it retains the majority of its polyphenols and antioxidants (5). Oolong tea on the other hand is 10-80% oxidized, thus depending on the processing conditions, the TPC and TAC could be affected (28). Day 9 is of more interest here as well because of sensory properties and the TAC content either decreasing or remaining constant after this time point.

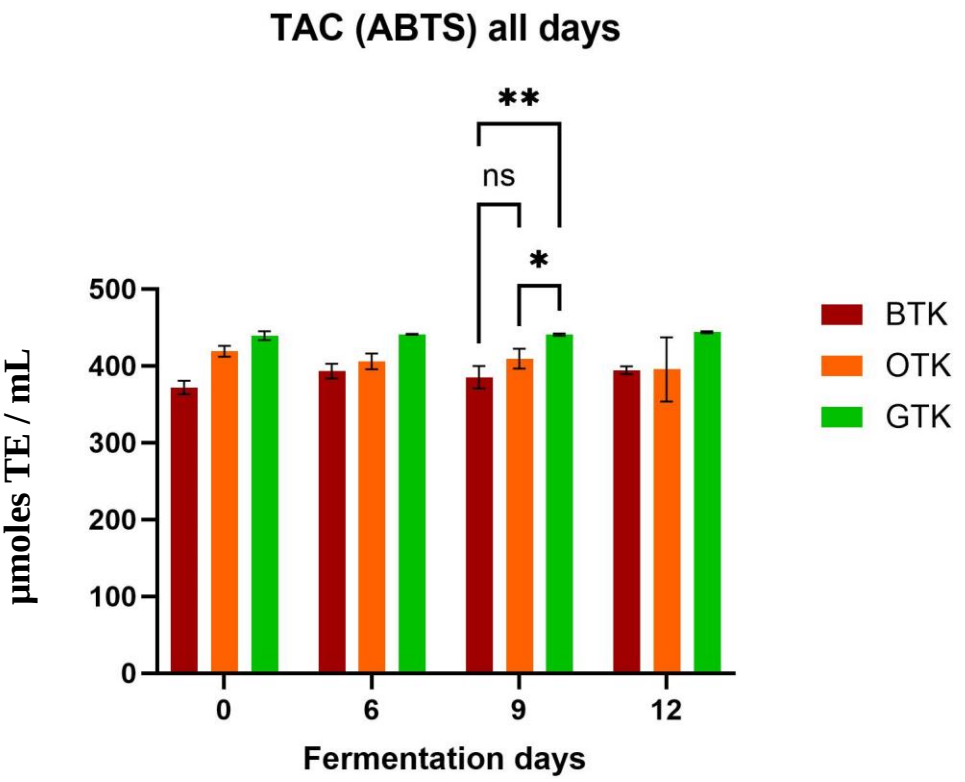

Figure 7 – TAC results by ABTS assay for black tea kombucha, oolong tea kombucha, and green tea kombucha as a function of fermentation days measured as µmoles TE / mL.

The difference in the TAC value obtained by these two methods for a particular type of kombucha on a specific fermentation day is because they work with different affinities toward hydrophilic groups. DPPH radical doesn't contain that and only contains lipophilic ions. Thus, it won't react with polar molecules if they are present in the sample. ABTS contains both hydrophilic and lipophilic ions and can react with both polar and non-polar molecules if present in the sample (29).

A reason why the values obtained by ABTS for BTK and OTK are so higher than DPPH could be because of the polyphenolic composition of black tea and oolong tea. Both Black tea and oolong tea contain a higher content of theaflavins and caffeine than green tea (30). These polyphenols are polar in nature and are thus not detected by DPPH ion but are detected by ABTS ion (31). On the other hand, green tea contains more catechins that have both polar and non-polar affinity and are thus detected by both DPPH and ABTS ions (30, 32).

By both methods, GTK had a significantly higher TAC value than BTK and OTK on day 9 (in general higher) and thus GTK on day 9 (GTK 9) was chosen for performing studies on the intestinal epithelial tissue model. Since polyphenols are one of the major antioxidants for kombucha along with acetic acid or glucuronic acid, the TAC is directly related to the TPC. This provides a good indication to find out which method works the best for which type of kombucha. For BTK and OTK, ABTS results correlate with the TPC better than DPPH (R^2 = 0.7441 and 0.5783 respectively), but for GTK, both DPPH comparatively correlates the most (R^2 = 0.1787)

4.5 Intestinal epithelial tissue model (SMI-100)

The tissue re-equilibration and incubation were done successfully. While sterilizing the green tea kombucha sample by autoclaving at 121ºC, the lid of the test tube was deformed so to ensure the sample was completely sterile, filter sterilization with a 0.2 µm filter was done as well. The treatment took place successfully as well. Figure 8 contains a schematic representation of how the tissue insert and the tissue plate (22).

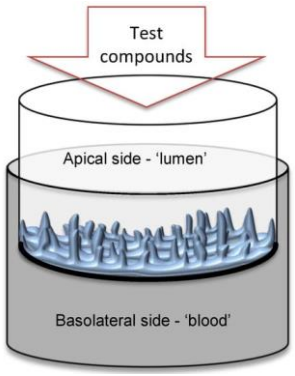

Figure 8 – Schematic diagram of a tissue insert in the SMI-100 model.
Source - (Kostiuchenko et al., 2022) (22)

4.6 Transepithelial electrical resistance (TEER)

Transepithelial electric resistance tells us about tissue integrity and permeability. In this case, the aim was to measure the TEER of the tissue inserts after exposing them to 4 different treatments and comparing the results with the negative control. The data obtained from the TEER measurement is summarized in Figure 9.

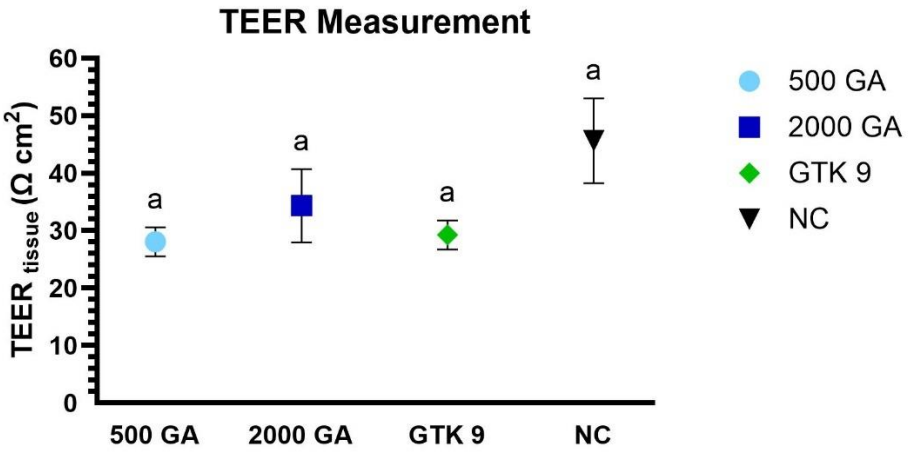

Figure 9 – TEER measurement for different tissue inserts with treatments of gallic acid (500 µg/mL), gallic acid (2000 µg/mL), green tea kombucha sample at day 9, and negative control.

The same small letter near each treatment indicates no significant difference ($p>0.05$) for the samples compared to the negative control.

The TEER results for 500 GA, 2000 GA, and GTK 9 tissues were not significantly different than the negative control, as seen in Figure 9 but it was noticed that the TEER value for the later treatment is 3 times less than the study conducted by Kostiuchenko et al. (2022) (22). One possible reason for this could be that the tissues were resting in the buffer for a longer time than they should have been, and this could have affected the readings as the tissues are very sensitive to external conditions (33).

4.7 Antioxidative capacity (AOC)

The data obtained by analysing the red colour intensity of the tissues by using a red colour probe is summarized in Table 1. The mean red colour intensity gives an indication of the reactive oxygen species (ROS) present in the tissue. The more the red colour intensity, the more are ROS and the less effective is that particular treatment.

Table 1 – Mean red colour intensity of tissues exposed to different treatments of gallic acid (500 µg/mL), gallic acid (2000 µg/mL), green tea kombucha sample at Day 9, and negative control during antioxidative capacity test.

Sample	Antioxidative capacity (Mean colour intensity)
500 GA	3434.14 ± 308.4 ****
2000 GA	1370.79 ± 299.03 ****
GTK 9	3341.09 ± 150.22 ****
NC	12512.21 ± 125.65

***** - Significant difference ($p<0.0005$) for the mean colour intensity measurement for different treatments compared to the NC.*

The negative control sample shows the highest mean colour intensity meaning the tissues were more affected by the induced oxidative stress generating ROS which oxidized the probe giving a bright red colour. Compared to the negative control, 500 GA, 2000 GA, and GTK 9 had significantly less ($p < 0.0005$) mean colour intensity. This was the least in the case of 2000 GA, indicating it is a good antioxidant for the tissues and the value was similar for 500 GA and GTK 9 as the latter contains a polyphenolic content similar to the former. Figure 10 (A-D) shows how the tissues looked under the microscope after different treatment exposures.

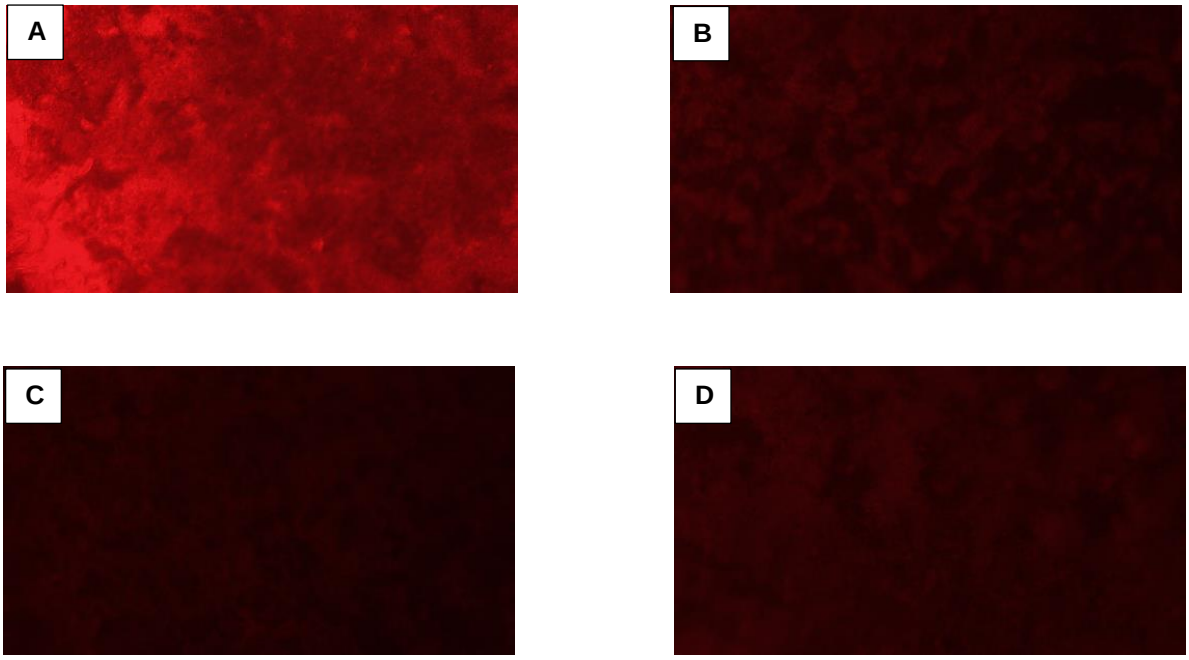

Figure 10 – Tissues observed under a microscope using 4x magnification and 1s exposure time after antioxidant assay on tissues exposed to (A) negative Control, (B) gallic acid (500 µg/mL), (C) gallic acid (2000 µg/mL) and (D) green tea kombucha sample at day 9.

Considering the mean colour intensity for the negative control to be 100%, the percentage mean colour intensity was calculated for 500 GA, 2000 GA, and GTK 9 and is depicted in Figure 11 in the form of a bar graph.

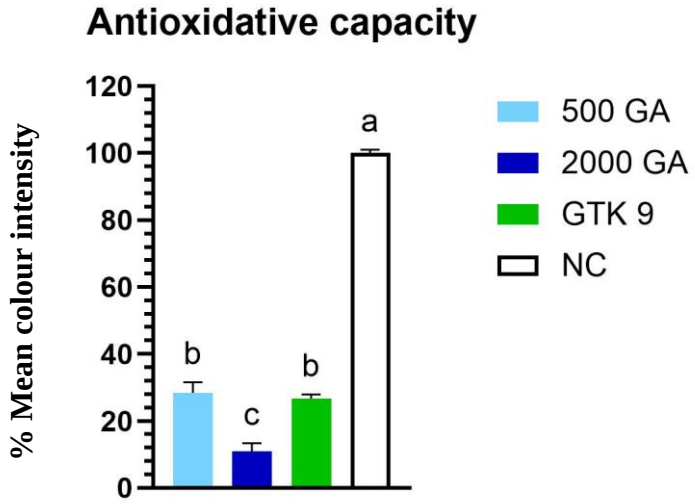

Figure 11 – % mean colour intensity of gallic acid (500 µg/mL), gallic acid (2000 µg/mL, green tea kombucha sample at day 9 compared to the negative control.

Different small letters show a significant difference ($p < 0.005$) compared to NC.

4.8 Permeability assay

Results obtained from the permeability test are summarized in Figure 12. Compared to the negative control, 2000 GA and GTK 9 had a higher % uptake of lucifer yellow passage from the apical to basolateral side whereas the difference was not significant in the case of GA 500, as depicted in Figure 12. This means that 500 GA did not change the tissue permeability compared to the negative control, but it increased when the tissues were exposed to 2000 GA and GTK 9. Nonetheless, this increase is within the acceptable range which is 1% for the SMI-100 model below which the tissues are healthy, and its permeability and integrity are strong enough.

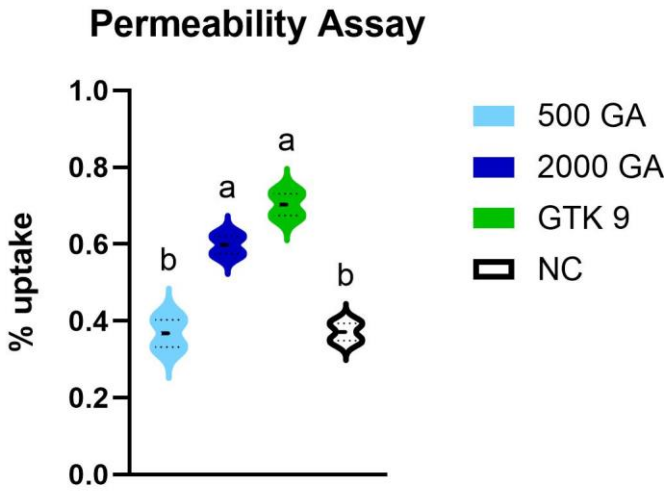

Figure 12 – Results obtained from the permeability assay of tissues exposed to gallic acid (500 µg/mL), gallic acid (2000 µg/mL), green tea kombucha sample at day 9, and negative control expressed as % uptake of LY from the apical side to the basolateral side.

Different small letters show a significant difference ($p < 0.005$) compared to NC.

LY molecule has a size of 452 Da (22), this molecule is very small compared to bacteria. The passage of LY from the apical to the basolateral side indicates that the cellular integrity is weakening, which means the tight junctions holding the cells together are developing some gaps. If the concentration of LY in the basolateral side is high or if the % uptake is more than 1% then that means the tissue is becoming permeable and might indicate the onset of a leaky gut or inflammatory bowel disease (16,19). On the other hand, some increase in permeability is good as it might allow the absorption of certain molecules like essential amino acids and improve their bioavailability for humans (13).

4.9 MTT assay

The principle of this assay is that MTT salt, a yellow colour salt gets converted to formazan, a violet colour dye in the presence of mitochondrial reductase. Mitochondrial reductase is produced by living cells. So, if the cells are living meaning the % viability is more than 100% then more violet colour compound will be produced during extraction indicating the treatment is not cytotoxic. On the contrary, if the cells are dying, meaning the % viability is less than 100% then a less violet colour compound will be produced during extraction indicating the treatment is cytotoxic.

This trend is more clear from Figure 13 where the negative control has kept the % viability above 100%. Compared to the negative control, 500 GA, 2000 GA, and GTK 9 do not vary significantly and all had % viability more than 100% which tells us that these treatments promote cell growth and are not cytotoxic. Triton X100, a known cytotoxic compound had decreased the % cell viability to 12 %.

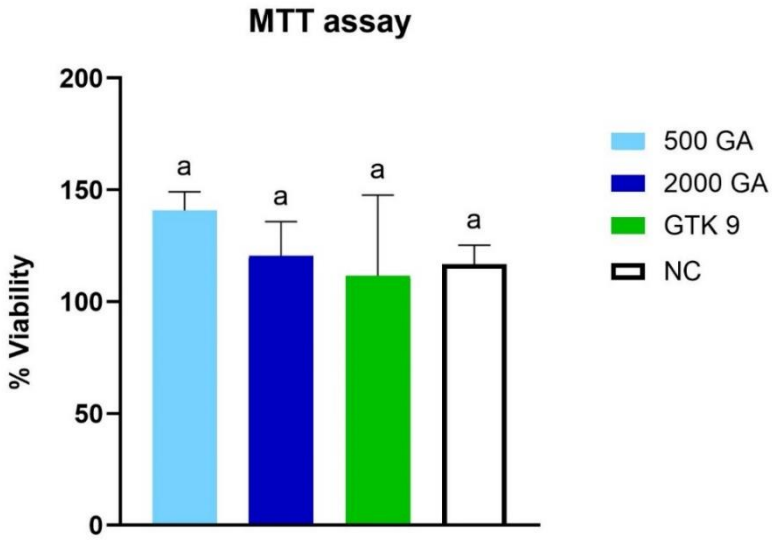

Figure 13 – Results from MTT assay of tissues exposed to gallic acid (500 µg/mL), gallic acid (2000 µg/mL), green tea kombucha sample at day 9, and the negative control expressed as % viability.

The same small letter near each treatment indicates no significant difference ($p>0.05$) for the samples compared to the negative control.

5. CHALLENGES AND FUTURE WORK

While this project provided some very interesting results, some of which were expected and some were not, it is worth mentioning certain drawbacks faced. The pH and TSS measurements showed large deviations after day 12 of fermentation for all types of kombucha. It could be interesting in the future to start fermenting the replicates of a type of kombucha on different days to get more reliable results and reduce the possibility of batch effect. The pellicle stored from the kombucha jars prepared in this project could be evaluated to figure out if something went wrong in a particular jar.

Folin- Ciocalteu assay is commonly used for TPC measurement, but it contains some drawbacks like it is very sensitive to pH and reaction time. Overestimation of TPC is a critical issue as well (34). This could have affected the results and its reproducibility. The TAC was measured by two methods, ABTS and DPPH which gave significantly different results because of different working principles. For further research, it would be drawing to use assays that have the similar working principle and compare the results. Samples could be taken every day after day 6 to create a better trend of change of TPC and TAC in the kombucha samples. It could be worthwhile conducting polyphenol profiling by HPLC to see the different proportions of polyphenols in the kombucha samples as a function of fermentation time.

The experiments conducted with the SMI-100 model were done under time constraints. Thus, some tissue inserts were exposed to the treatment for more than 24 hours, and some were kept in PBS (-Mg, Ca) for a longer time than they should have been. This could have created stress on the tissues and thus the results could have been affected for some tissues. It would be definitely interesting to have more replicates for a particular treatment, compare the kombucha at the start of fermentation and at the end, and have a better timeline for experiments to get better data for statistics and results.

Performing *in-vitro* digestion on the kombucha samples and then evaluating the effect of digested kombucha on the intestinal epithelial tissues is a future scope too. Since kombucha is a low pH food, checking the pH of all the standard solutions could be interesting to see if the results obtained are pH dependent or not.

6. CONCLUSION

3 types of kombucha made from black tea, oolong tea, and green tea in 4 repetitions each were prepared and fermented at Roots of Malmö. The pH and TSS decreased over the fermentation time from pH around 4.4 on day 0 to around 2.9 on day 18 and TSS around 5.7 on day 0 to 5 on day 18. The TPC measured by the Folin-Ciocalteu method did not decrease significantly ($p > 0.05$) over the course of fermentation for all types of kombucha. Amongst the kombucha samples, GTK had significantly higher ($p > 0.05$) TPC till day 9 and OTK had significantly lower ($p > 0.05$) TPC till day 18. The highest TPC was found in GTK 9 which was 506.97 ± 22.28 µg GAE/mL.

TAC by DPPH and ABTS was conducted on all samples till day 12. BTK and OTK had significantly lower ($p < 0.005$) TAC values by DPPH compared to ABTS, whereas the values for GTK were not significantly different ($p > 0.05$) by the two methods. The difference in values obtained by these two methods is because of their different affinities. The TAC values measured by DPPH did not increase significantly over the fermentation time period for BTK and GTK but for OTK, the value increased significantly ($p < 0.05$) on day 18 compared to day 0. The increase was insignificant for TAC values measured by ABTS in all samples. Between the types of kombuchas, GTK had a significantly higher TAC value by both DPPH and ABTS methods, and a maximum on day 9 by DPPH (449.46 ± 8.01 µmoles TE/mL).

Because of high TPC and TAC, GTK 9 was selected for experiments on the intestinal epithelial tissue model along with 500 µg/mL GA, 2000 µg/mL GA, and NC treated with PBS. TEER of all the treatments was not significantly lower ($p > 0.05$) than the NC. The AOC of the tissues treated with 2000 GA, 500 GA, and GTK 9 decreased significantly ($p < 0.0005$) compared to NC. 2000 GA treated tissues had only 10% of oxidized tissues and 500 GA and GTK 9 had around 26% compared to NC which had 100%. Tissue permeability was significantly increased ($p<0.05$) by GTK followed by 2000 compared to NC but this increase was within the acceptable range of 1% to be harmful for the tissues. None of the treatments proved to decrease % viability and had cytoprotective effects for the intestinal epithelial tissues.

Kombucha, especially made with green tea and gallic acid, a simple phenolic compound both proved to have improved the intestinal epithelial tissue health compared to the NC because of high TPC and TAC content.

REFERENCES

1. Batista P, Penas MR, Pintado M, Oliveira-Silva P. Kombucha: Perceptions and future Prospects. Foods [Internet]. 2022 Jul 4;11(13):1977. Available from: https://www.mdpi.com/2304-8158/11/13/1977

2. Suna S, Çiftçi K, Tamer CE. SAFRAN KULLANILARAK HAZIRLANAN KOMBUCHA İÇECEĞİNİN FİZİKOKİMYASAL VE DUYUSAL ÖZELLİKLERİNİN BELİRLENMESİ. Gıda [Internet]. 2019 Dec 27;20–30. Available from: https://doi.org/10.15237/gida.gd19139

3. Özyurt VH. Changes in the content of total polyphenols and the antioxidant activity of different beverages obtained by Kombucha 'tea fungus.' International Journal of Agriculture, Environment and Food Sciences [Internet]. 2020 Sep 15;4(3):255–61. Available from: https://doi.org/10.31015/jaefs.2020.3.3

4. Sutthiphatkul T, Mangmool S, Rungjindamai N, Ochaikul D. Characteristics and Antioxidant Activities of Kombucha from Black Tea and Roselle by a Mixed Starter Culture. Current Applied Science and Technology [Internet]. 2022 Oct 29;23(4). Available from: https://doi.org/10.55003/cast.2022.04.23.002

5. Wang X, Wang DH, Wang H, Jiao S, Wu J, Hou Y, et al. Chemical profile and antioxidant capacity of Kombucha Tea by the pure cultured Kombucha. Lebensmittel-Wissenschaft + Technologie/Food Science & Technology [Internet]. 2022 Oct 1;168:113931. Available from: https://doi.org/10.1016/j.lwt.2022.113931

6. Jayabalan R, Malbaša RV, Lončar ES, Витас J, Sathishkumar M. A review on Kombucha Tea—Microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus. Comprehensive Reviews in Food Science and Food Safety [Internet]. 2014 Jun 21;13(4):538–50. Available from: https://doi.org/10.1111/1541-4337.12073

7. Değirmencioğlu N, Yıldız E, Şahan Y, Güldaş M, Gürbüz O. Impact of tea leaves types on antioxidant properties and bioaccessibility of kombucha. Journal of Food Science and Technology/Journal of Food Science and Technology [Internet]. 2020 Sep 1;58(6):2304–12. Available from: https://doi.org/10.1007/s13197-020-04741-7

8. Chen NX and ZM. GREEN TEA, BLACK TEA AND SEMI-FERMENTED TEA. In: CRC Press eBooks [Internet]. 2002. p. 47–68. Available from: https://doi.org/10.1201/b12659-6

9. Okatan V. Antioxidant properties and phenolic profile of the most widely appreciated cultivated berry species: A comparative study. Folia Horticulturae [Internet]. 2020 Apr 22;32(1):79–85. Available from: https://doi.org/10.2478/fhort-2020-0008

10. Santana MG, Freitas-Silva O, Mariutti LRB, Teodoro AJ. A review of in vitro methods to evaluate the bioaccessibility of phenolic compounds in tropical fruits. Critical Reviews in Food Science and Nutrition [Internet]. 2022 Sep 5;64(6):1780–90. Available from: https://doi.org/10.1080/10408398.2022.2119203

11. Sharma R, Diwan B, Singh BP, Kulshrestha S. Probiotic fermentation of polyphenols: potential sources of novel functional foods. Food Production, Processing and Nutrition [Internet]. 2022 Sep 5;4(1). Available from: https://doi.org/10.1186/s43014-022-00101-4
11. Huang Y, Xiudan X, Li C, Wu M, Huang Y, Yao Y. A fermented tea with high levels of gallic acid processed by anaerobic solid-state fermentation. Lebensmittel-Wissenschaft + Technologie/Food Science & Technology [Internet]. 2016 Sep 1;71:260–7. Available from: https://doi.org/10.1016/j.lwt.2016.03.047

13. Tretola M, Bee G, Silacci P. Gallic acid affects intestinal-epithelial-cell integrity and selected amino-acid uptake in porcine in vitro and ex vivo permeability models. British Journal of Nutrition [Internet]. 2020 Nov 4;126(4):492–500. Available from: https://doi.org/10.1017/s0007114520004328

14. Kahkeshani N, Farzaei F, Fotouhi M, Alavi SS, Bahramsoltani R, Naseri R, et al. Pharmacological effects of gallic acid in health and diseases: A mechanistic review. PubMed [Internet]. 2019 Mar 1;22(3):225–37. Available from: https://pubmed.ncbi.nlm.nih.gov/31156781

15. Jiang Y, Pei J, Zheng Y, Miao Y, Duan B, Huang L. Gallic Acid: a potential Anti-Cancer agent. Chinese Journal of Integrative Medicine/Chinese Journal of Integrative Medicine [Internet]. 2021 Nov 10;28(7):661–71. Available from: https://doi.org/10.1007/s11655-021-3345-2

16. Wijeratne SSK, Cuppett SL, Schlegel V. Hydrogen peroxide induced oxidative stress damage and antioxidant enzyme response in CACO-2 human colon cells. Journal of Agricultural and Food Chemistry [Internet]. 2005 Oct 1;53(22):8768–74. Available from: https://doi.org/10.1021/jf0512003

17. Wu Z, Huang S, Li T, Li N, Han D, Zhang B, et al. Gut microbiota from green tea polyphenol-dosed mice improves intestinal epithelial homeostasis and ameliorates experimental colitis. Microbiome [Internet]. 2021 Sep 7;9(1). Available from: https://doi.org/10.1186/s40168-021-01115-9

18. Yang G, Bibi S, Du M, Suzuki T, Zhu M. Regulation of the intestinal tight junction by natural polyphenols: A mechanistic perspective. Critical Reviews in Food Science and Nutrition [Internet]. 2016 Mar 23;57(18):3830–9. Available from: https://doi.org/10.1080/10408398.2016.1152230

19. Teshima C, Meddings J. The measurement and clinical significance of intestinal permeability. Current Gastroenterology Reports [Internet]. 2008 Oct 1;10(5):443–9. Available from: https://doi.org/10.1007/s11894-008-0083-y

20. Zhang Y, Wei M, Zhang F, Guo J. High-accuracy gastric cancer cell viability evaluation based on multi-impedance spectrum characteristics. Heliyon [Internet]. 2023 Apr 1;9(4):e14966. Available from: https://doi.org/10.1016/j.heliyon.2023.e14966

21. Li Y, Park HJ, Xiu H, Akoh CC, Kong F. Predicting intestinal effective permeability of different transport mechanisms: Comparing ex vivo porcine and in vitro dialysis models. Journal of Food Engineering [Internet]. 2023 Feb 1;338:111256. Available from: https://doi.org/10.1016/j.jfoodeng.2022.111256
22. Kostiuchenko O, Kravchenko NA, Markus J, Burleigh S, Fedkiv O, Cao L, et al. Effects of Proteases from Pineapple and Papaya on Protein Digestive Capacity and Gut Microbiota in Healthy C57BL/6 Mice and Dose-Manner Response on Mucosal Permeability in Human Reconstructed Intestinal 3D Tissue Model. Metabolites [Internet]. 2022 Oct 26;12(11):1027. Available from: https://doi.org/10.3390/metabo12111027

23. MatTek Corporation. EpiIntestinal 3D in vitro Microtissues | MatTek Life Sciences [Internet]. MatTek Life Sciences. 2023. Available from: https://www.mattek.com/mattekproduct/epiintestinal (Accessed: 29 April 2024)

24. World Precision Instruments | Cell & Tissue | TEER Measurement | EVOM2 [Internet]. Available from: https://www.wpi-europe.com/products/cell-and-tissue/teer-measurement/evom2.aspx (Accessed: 30 April 2024)

25. Raspor P, Goranovič D. Biotechnological applications of acetic acid bacteria. Critical Reviews in Biotechnology [Internet]. 2008 Jan 1;28(2):101–24. Available from: https://doi.org/10.1080/07388550802046749

26. Wang B, Rutherfurd-Markwick K, Naren N, Zhang X, Mutukumira AN. Microbiological and Physico-Chemical Characteristics of Black Tea Kombucha Fermented with a New Zealand Starter Culture. Foods [Internet]. 2023 Jun 8;12(12):2314. Available from: https://www.mdpi.com/2304-8158/12/12/2314

27. Velićanski A, Cvetković D, Markov S, Šaponjac VT, Vulić J. Antioxidant and Antibacterial Activity of the Beverage Obtained by Fermentation of Sweetened Lemon Balm (Melissa officinalis L.) Tea with Symbiotic Consortium of Bacteria and Yeasts. Food Technology and Biotechnology [Internet]. 2014 Dec 19;52(4):420–9. Available from: https://pubmed.ncbi.nlm.nih.gov/27904315/

28. Weerawatanakorn M, Hung WC, Pan M, Li S, Li D, Wan X, et al. Chemistry and health beneficial effects of oolong tea and theasinensins. Food Science and Human Wellness [Internet]. 2015 Dec 1;4(4):133–46. Available from: https://www.sciencedirect.com/science/article/pii/S2213453015000555

29. Floegel A, Kim D, Chung S, Koo SI, Chun OK. Comparison of ABTS/DPPH assays to measure antioxidant capacity in popular antioxidant-rich US foods. Journal of Food Composition and Analysis [Internet]. 2011 Nov 1;24(7):1043–8. Available from: https://doi.org/10.1016/j.jfca.2011.01.008

30. Salman S, Öz G, Felek R, Haznedar A, Turna T, Özdemir F. Effects of fermentation time on phenolic composition, antioxidant and antimicrobial activities of green, oolong, and black teas. Food Bioscience [Internet]. 2022 Oct 1;49:101884. Available from: https://doi.org/10.1016/j.fbio.2022.101884

31. Morlock GE, Heil J, Inarejos-García AM, Maeder J. Effect-Directed profiling of powdered tea extracts for catechins, theaflavins, flavonols and caffeine. Antioxidants [Internet]. 2021 Jan 15;10(1):117. Available from: https://www.mdpi.com/2076-3921/10/1/117

32. Tsuchiya H. Stereospecificity in membrane effects of catechins. Chemico-biological Interactions [Internet]. 2001 Mar 1;134(1):41–54. Available from: https://doi.org/10.1016/s0009-2797(00)00308-2

33. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER measurement techniques for in vitro barrier model systems. SLAS Technology [Internet]. 2015 Apr 1;20(2):107–26. Available from: https://doi.org/10.1177/2211068214561025

34. Munteanu IG, Apetrei C. Analytical methods used in Determining antioxidant Activity: a review. International Journal of Molecular Sciences [Internet]. 2021 Mar 25;22(7):3380. Available from: https://doi.org/10.3390/ijms22073380

APPENDIX

A1. pH and TSS values obtained for samples at Roots of Malmö

pH was measured using water analysis meter PCE-PH 22 (PCE instruments) and the TSS was measured using Brix refractometer (PCE instruments).

Table A1 – pH and TSS values for starter culture, BTK, OTK and GTK at the start and end of fermentation

Samples		Starter culture	BTK	OTK	GTK
Day 0	**pH**	2.8	4.1	4.03	4.02
	TSS (ºBrix)	4.5	5.75	5.5	5.6
Day 18	**pH**	-	2.93	2.91	2.99
	TSS (ºBrix)	-	4.9	4.8	4.8

A2. Pure teas and culture data

Table A2. pH, TSS, TPC and TAC (DPPH and ABTS) values for the starter culture, black tea, oolong tea and green tea

Samples	Starter culture	Black tea	Oolong tea	Green tea
pH	3.17	6.83±0.06	6.83±0.15	6.85±0.06
TSS (ºBrix)	3.9	6±0.08	5.8±0.08	5.85±0.06
TPC (µg GAE/mL)	341.09 ± 30.51	357.56±12.84	316.59±18.42	429.34±11.51
TAC (DPPH) (µmoles TE/ mL)	944.55±0	638.39±67.23	663.99±325.81	550.1±10.36
TAC (ABTS) (µmoles TE/ mL)	118.76±0	363.1±9.63	399.97±6.11	438.69±0.64

A3. Cellulose pellicles

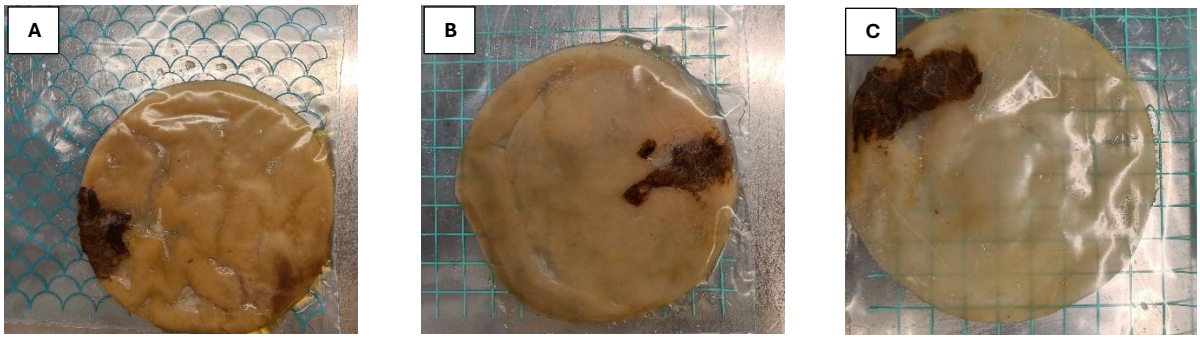

Figure A3- Cellulose pellicles formed in the fermentation jars of
A – black tea kombucha, B – oolong tea kombucha and C- green tea kombucha at the end of day 18

A4. ABTS vs DPPH on day 9

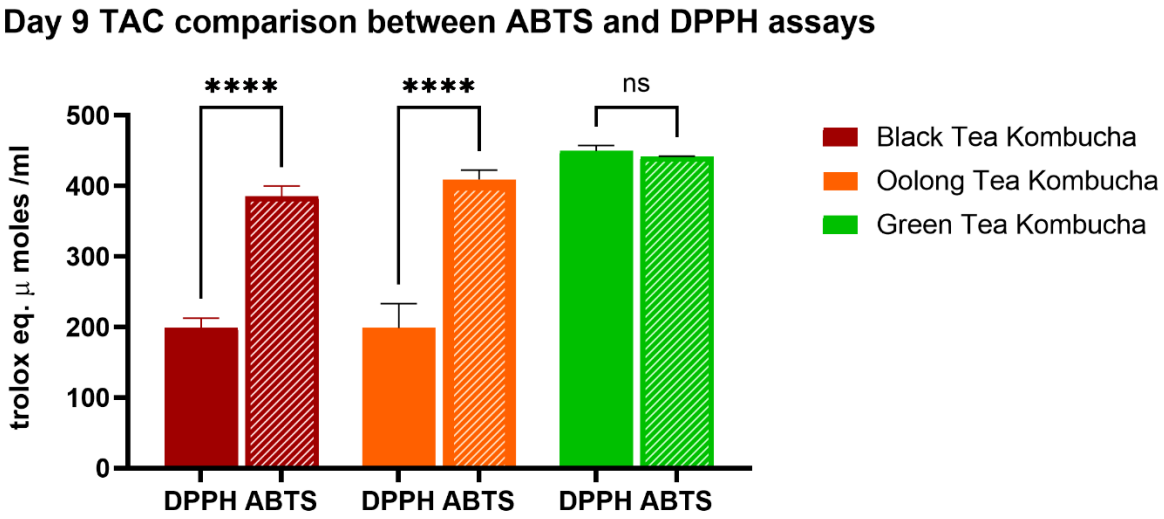

Figure A4. TAC comparison between the TAC values obtained by DPPH and ABTS assays for BTK, OTK and GTK for day 9 samples expressed as µmoles TE/ mL.