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Graduation Research Project

Development of a Fluorescence-Based Method to Estimate Bacterial Density in In Vitro Fermentation Studies

Presented By

Ana Victoria Troncoso Saavedra

Advisers

Miguel Alvarez Gonzales

Stephen R. Lindemann

Elizabeth Ligia Luna Jarrin

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Authorities

SERGIO ANDRÉS RODRÍGUEZ ROYO

President

ANA M. MAIER ACOSTA

Vice President and Academic Dean

ADELA M. ACOSTA MARCHETTI

Head of Food Science and Technology Department

HUGO ZAVALA MEMBREÑO

General Secretary

Content

List of Tables

List of Figures

Abstract

Human gut microbiota composition, structure, and density varies among individuals mainly due to age, eating habits, gender, and other factors. Although human microbial biodiversity varies between individuals with respect to species composition, variable fecal bacterial load may introduce an artifact during *in vitro* fermentations, resulting in variable fermentation outcomes in terms of metabolite production and composition and structure of the microbiota. Multiple methods have been developed for the estimation of bacterial density; however, these tend to require considerable time to obtain results and/or expensive equipment, making their use difficult for measurement of bacterial density in inoculum before *in vitro* experiments. Here, we describe a convenient and cost-effective method to estimate bacterial density from diluted stool by staining bacteria with a fluorophore and measuring its fluorescence intensity. For our preliminary test, *Escherichia coli* cells were stained with SYBR Gold, SYBR Green, and DAPI to identify the fluorophore with the highest fluorescence intensity. SYBR Gold was selected as the best fluorophore and the method was optimized by maintaining the sample wash step and fluorophore concentration. Fluorescence intensity measurements were collected on a microplate reader to assess staining efficiency and cell numbers were estimated using a hemacytometer. Finally, the method was used to estimate the bacterial density in diluted feces obtained from two donors to later use this method to test the effect of normalizing the fecal bacterial load before in vitro fermentation.

Keywords: bacterial density; fluorophore; normalization; microbiota.

Resumen

La composición, estructura y densidad de la microbiota del intestino humano varía entre individuos principalmente debido a factores como la edad, los hábitos alimenticios, el género y otros determinantes. La biodiversidad microbiana humana difiere entre sujetos en términos de la composición de especies, por lo que la variabilidad en la carga bacteriana fecal puede introducir un sesgo durante las fermentaciones in vitro, lo que conlleva a resultados variables en lo que respecta a la producción de metabolitos y la composición y estructura de la microbiota. Se han desarrollado múltiples métodos para estimar la densidad bacteriana, estos tienden a requerir un tiempo considerable para obtener resultados y/o equipos costosos, lo que dificulta su implementación para medir la densidad bacteriana en el inóculo previo a los experimentos in vitro.

En este trabajo, describimos un método conveniente y económico para estimar la densidad bacteriana a partir de heces diluidas mediante la tinción de bacterias con un fluoró foro y la medición de su intensidad de fluorescencia. Para nuestra prueba preliminar, las células de Escherichia coli se tiñeron con SYBR Gold, SYBR Green y DAPI para identificar el fluoró foro con la mayor intensidad de fluorescencia. Se seleccionó SYBR Gold como el mejor fluoró foro y el método se optimizó manteniendo el paso de lavado de la muestra y la concentración de fluoró foro. Las mediciones de intensidad de fluorescencia se recopilaron en un lector de microplacas para evaluar la eficiencia de tinción y se estimaron los números de células utilizando un hemocitómetro. Finalmente, el método se utilizó para estimar la densidad bacteriana en heces diluidas obtenidas de dos donantes para luego utilizar este método para probar el efecto de normalizar la carga bacteriana fecal antes de la fermentación in vitro.

Palabras clave: Densidad bacteriana, fluoró foro, microbiota, normalización.

Introduction

This study aimed to develop a method to estimate colonic bacterial density using a fluorophore and normalize bacterial density prior to *in vitro* batch fermentations in the study of the gut microbiome. The human gut microbiome is one of the most abundant and complex bacterial communities with approximately 3,500 bacterial species (Frank et al., 2007). Bacteroidetes, Firmicutes, Actinobacteria, Proteobacteria and Verrucomicrobia represent more than 90% of the bacterial phyla (Donaldson et al., 2016) and can be found in different sections of the human intestinal tract. Several factors affect the composition, structure, and function of the gut microbiome and the most important being diet, gender, age, and the genetic diversity of the individual (Thursby y Juge, 2017).

The gut microbiome utilizes complex carbohydrates, e.g. dietary fiber, which reach the intestinal tract, to produce short chain fatty acids: acetate, propionate, and butyrate. Acetate is the most abundant SCFA produced, acting as a cofactor and essential metabolite for the growth of other bacteria (Rowland et al., 2018); propionate is a source of energy for epithelial cells; and butyrate, which serves as an energy source for colonocytes, inhibits the growth of pathogens, maintains the intestinal barrier (Jandhyala et al., 2015), and is used in the metabolism of bile acids and synthesis of vitamin K, B12, biotin, folic acid, and pantothenic acid (Álvarez et al., 2021).

Microbiome studies are primarily conducted to understand how our diet impacts the composition, structure, and function of microbial communities in the human gut. *In vitro* fermentation models, which simulate sections of the intestinal tract, are used as a tool to understand the impact of foods on our microbial ecosystems. In an *in vitro* fermentation setting, different substrates of interest are inoculated with fecal samples from healthy donors, allowing the study of microbial processes, metabolic pathways, microbial degradation of the substrates, and production of metabolites (Venema y van den Abbeele, 2013).

Batch *in vitro* fermentations are considered the closest simulation of physiological conditions and processes in the colon for studying microbiome responses to different substrates by elucidating

the different metabolic pathways involved during fermentation, metabolites produced, and their effect in our health (Pérez-Burillo et al., 2021).

Previous results in *in vitro* fermentations have shown that there is a need to normalize bacterial density before conducting fermentation experiments. This is due to strong donor effects that resulted in variable fermentation outcomes in terms of bacterial communities and metabolite production. These variations have introduced uncertainties in the obtained results. Therefore, it is proposed to normalize the microbiota density before the fermentation experiments.

To achieve this normalization, it is necessary to know the initial bacterial density of each sample. Several methods are currently available to estimate cell density, including the Neubauer chamber, automated cell counters, manual counting methods, flow cytometry methods based on autofluorescence, plate counting, and others. However, most natural communities cannot be accurately enumerated as CFU by culturing on various agar media (Lebaron et al., 1998). While some of these methods are accurate, many of them require a considerable amount of time to yield results and/or expensive equipment. This makes their application for normalizing inoculum in *in vitro* experiments challenging. Additionally, it is important to consider the time and initial investment required.

One of the traditional methods is manual counting using a hemocytometer. This technique involves counting cells within a specific volume using a grid on a specialized glass slide. Although accurate, it demands a considerable time and skill to achieve accurate counts.

Automatic cell counters use an image analysis algorithm that automatically identifies and enumerates particles present in images, and the system calculates their density per area and unit volume, based on parameters previously set during system calibration. They often use dyes such as trypan blue and propidium iodide to distinguish between living and dead cells. These automatic counters provide fast and accurate results with minimal user intervention; however, their acquisition cost can be high (Camacho-Fernández et al., 2018).

Flow cytometry is a powerful technique used for cell counting and cell density estimation. Cells are labeled with fluorescent dyes or antibodies and passed through a flow cytometer, which can analyze thousands of cells per second based on their size, granularity, and fluorescence properties. By measuring the passage rate of the cells, the total cell count can be estimated (Amal A E Ibrahim, 2019).

Hemacytometers are the most reasonable option for cell counting, which may explain their widespread use. Automated cell counters also show a good correlation between precision and affordability, albeit with limited accuracy. Flow cytometry methods are the best in terms of reproducibility and agreement between them, but they showed deficient accuracy and precision (Weiss et al., 2017).

It has been observed that an increase in cellular constituents such as proteins and nucleic acids can serve as a reliable marker of biomass production during microbial growth. Therefore, determining the bulk nucleic acid content in microbial cultures is likely to provide a sensitive and reliable tool to assess microbial growth (Martens-Habbena y Sass, 2006).

For this reason, we propose a method to measure the total bacterial load in diluted stools using a fluorophore.

The aims of this study were:

Develop a protocol for the normalization of bacteria cell load in diluted fecal samples, assess the existence of a correlation between the number of bacteria and fluorescence intensity and evaluate the developed protocol by applying it at beginning of a fermentation process.

Materials and methods

Study Location

This study was development in the Diet-Microbiome Interactions Laboratory (D-MIL) at Purdue University.

Bacterial Strains and Culture Conditions

The initial analysis was performed using a facultative anaerobic bacterium: *Escherichia coli*, which is commonly found in the lower intestine (Sri Chandana Panchangam, 2015). This was followed by the inclusion of anaerobic bacteria: *Bifidobacterium longum, Einsergebella tayi MKS and Clostridium cochlearium* which are members of the human colonic microbiota. The strains were obtained from the Diet-Microbiome Interactions Laboratory (D-MIL) at Purdue University. The bacteria were cultivated in 1X phosphate buffer (1X FB), which was prepared following a previously described method with a slight modification (Yao et al., 2020). The buffer was filtered using a 0.22-µm filter in place of the autoclave step.

The bacteria *B. longum, E. tayi, and C. cochlearium* are colonic bacteria that exhibit different genome sizes (Table 1). A test was conducted to understand whether these differences in genome size influence fluorescence intensity.

Table 1

Genome size, taxonomy, and Gram stain of the bacterial strains used in this study.

Name	Genome size (Mb)	Taxonomy	Gram stain	Reference
E. coli	4.5 to 5.5	Enterobacteriaceae	Negative	(Rode et al., 1999)
Bifidobacterium longum	± 2.44	Bifidobacteriaceae	Positive	(Andryuschenko et al., 2019)
Einsergebella tayi	7.1-8.3	Lachnospiraceae	Positive	(Bernard et al., 2017)
Clostridium cochlearium	2.7	Clostridiaceae	Positive	(Gupta et al., 2020)

Preliminary Test

During this phase, four fluorophores were tested to choose the fluorophore with higher fluorescence intensity. The fluorophores were obtained from Fisher Scientific: SYBR Gold, SYBR Green I, SYTO BC, and DAPI (Table 2).

Table 2

Values in nm of emission and excitation of the fluorophores

DYE	Excitation (nm)	Emission (nm)
SYBR Gold	∽490	∽525
SYBR Green	∽490	∽525
DAPI	∽358	∽461
SYTO BC	∽485	∽502

Fluorescence Intensity Measurements and Microscopy

To measure fluorescence intensity, Thermo Scientific™ Black 96-Well Immuno Plates (Fisher Scientific) were used. Fluorescence intensity measurements were performed using a microplate reader (Synergy H1, BioTek) using the software BioTek Gen5 ver. 3.12, and the excitation and emission wavelength described in Table 2.

Staining Procedure

The selected fluorophores for this experiment were used in accordance with the staining protocol available in the D-MIL at Purdue University, which had been modified based on the manufacturer's instructions. The sample was mixed with 50 µL of a 5,000X diluted solution of the fluorophores (Stock solution). Subsequently, the mixture was incubated for 30 minutes, followed by a centrifugation step at 8,000 RPM for 10 minutes. The supernatant was discarded, leaving only the pellet, and the pellet was resuspended in 200 µL of filtered and sterilized water.

a) Optimization of the fluorophore concentration was done by testing three dilutions of the SYBR Gold fluorophore: 5uL/mL (0.5%), 10uL/mL (1%), and 20uL/mL (2%).

b) Preliminary tests were performed with *E. coli* in an aerobic environment, which may not accurately represent the natural habitat of colonic bacteria, known to be anaerobic. In order to

investigate the suitability of the staining protocol, a test was conducted to determine if an anaerobic environment was necessary to effectively stain the bacteria of interest.

For this comparison, three bacterial strains: *E. coli, B. longum,* and *Clostridium cochlearium*, were selected. These strains were stained with SYBR gold using the developed method in both aerobic and anaerobic environments. The main objective was to assess any potential differences in the fluorescence intensity of the stained bacteria between the two environments.

Permeabilization Procedure

To enhance bacterial staining, an organic solvent, ethanol, and three surfactants, Tween 20, Tween 80, and Triton X100, were tested to improve cell membrane permeabilization. These reagents were purchased from Fisher Scientific.

Purification Of Colonic Bacteria

Three methods to purify colonic bacteria were tested: 1) using a syringe filter with a pore size of 5-µm (Fisher Scientific), 2) centrifugation at 1,000 RPM for five min, and 3) centrifugation at 1,000 RPM for 10 min. After the purification of colonic bacteria, one mL was transferred to a 2-mL centrifuge tube.

Estimation Of Bacterial Cell Number

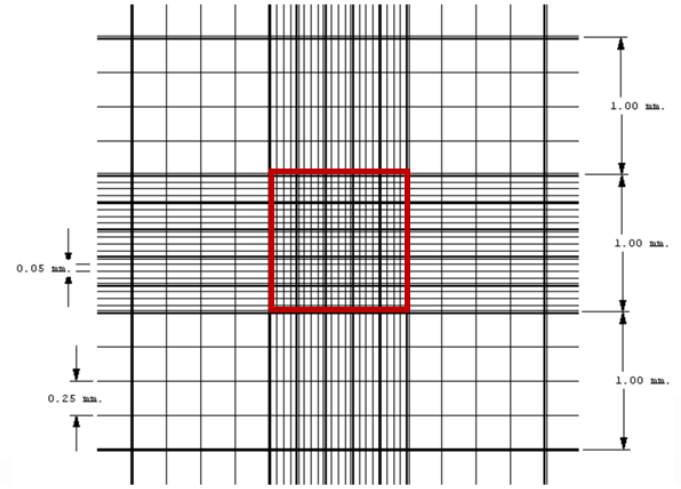
The number of bacterial cells was determine using the Petroff-Hausser counter (Hausser Scientific) with a cell-depth of 0.02mm (1/50 mm). A volume of 20 µL of bacterial suspension was added to the counting chamber, and the number of bacterial cells within the center square millimeter was counted. The bacterial density per milliliter was estimated using Equation 1, considering the squares in the middle of the counting chamber with the provided dimensions (Figure 1).

The equation [1] was used to determine cell density (cell/mL) using the Hemocytometer.

$$Concentration(\frac{cells}{ml}) = \frac{number\ of\ cells}{counting\ surface\ (mm2) * depth\ of\ the\ chamber(mm) * dilution} \quad [1]$$

Figure 1

Chamber dimensions of Petroff-Hausser Counter ((Haussers, 2021)



The Hemocytometer Sidekick application (Bauer 2020) was used as support to obtain the cell count, since this application operates based on Equation 1 and allows us to enter the dilution used, the depth of the chamber, and the number of cells observed in each Petroff-Hausser grid (Figure 1); so, it helps to calculate the results more efficiently.

Normalization Test

Carbohydrate Substrates

Wheat bran was received as a donation from Mennel Milling Co and 2 kg were sieved. Particles over 1.7 mm were collected and smaller particles sizes were discarded.

Wheat bran arabinoxylan was isolated from coarse wheat bran particles previously collected using alkali extraction followed by ethanol precipitation method (Rumpagaporn et al., 2015). A sugar control, Fibersol 2, was purchased from ADM and included in the experiment as positive control. Finally, a blank was also included as negative control, 1X FB with no substrate.

Sample Collection

Fecal inoculum was prepared anoxically as previously described (Yao, Chen, & Lindemann, 2020). Fecal sample from three healthy donors were obtained using a customized stool collection kit and then the samples were transferred into 50 mL Falcon tubes and placed in an anaerobic chamber

where each sample was separately diluted with 30 mL of 1X FB in a sterilized beaker and filtered through cheesecloth to remove coarse particles.

Purification Procedure

The diluted fecal sample was centrifuged at 1,000 RPM for 10 min to precipitate the rest of the small fiber particles, any other foreign compounds, and purify the bacteria of interest.

Permeabilization Procedure

Ethanol, 181.42 µl (0.55M) was added to one mL of bacteria isolated from donor fecal samples, the solution was mixed in the vortex for 15 seconds at 3,000 RPM and incubated at room temperature for 10 minutes. After incubation, the mixture was centrifuged at 11,000 RPM for 10 min at room temperature, and 881.42 µl of supernatant was discarded. The bacterial pellet was resuspended in 700 µl of miliQ® water.

Staining Procedure

Fifty µl of stock solution of SYBR gold was added to 1 mL of bacteria after the permeabilization step and incubated at room temperature for 45 minutes. After incubation, the mixture was centrifuged at 11,000 RPM for 10 min and 700 µl of supernatant was discarded. The stained bacterial pellet was resuspended in 700 µl of miliQ® water, the mixture was centrifuged at 11,000 RPM for 10 min, and 820 µl of supernatant was discarded.

Normalization Process

Two hundred µL of SYBR-gold-stained bacteria were transferred to a well of a black 96-well plate and fluorescence intensity was measured. After this, the bacterial cell number was estimated. Once the estimated bacterial density was known, samples in the anaerobic chamber were diluted with fortified buffer, taking as reference the sample that presented the lowest fluorescence intensity.

In Vitro Consecutive Fermentation

Normalized Fecal bacterial samples were compared to current lab practices (control) using in-vitro fermentation of two substrates (wheat bran and AX). Three fecal donors were enrolled in the study. The in vitro fermentation was performed in an anaerobic chamber with the maximum hydrogen

level set to 3% (Yao et al., 2020). The chamber was supplied with a gas tank with the following gas mixture 90% N2, 5% CO2, and 5% H2.

Fermentation of substrates was performed in Balch tubes which were sealed with butyl rubber stoppers and aluminum seals, and then incubated outside the chamber in a shaking incubator at 37 degrees Celsius with a shaking speed of 150 rpm. At each designated time point (3, 6, 12, 24, and 48 hours), the culture tubes were removed from the incubator. pH (Mettler Toledo ph-meter) and gas measurements were taken following the previously described method (Tuncil et al. 2018). Gas measurement was measured by overpressure using an 18-gauge needle attached to a pressure gauge. Then, the tubes were unsealed, and two samples were taken and stored at -80 degrees Celsius for subsequent analysis of short-chain fatty acids (SCFAs) and DNA extraction.

Statistical Analysis and Experimental Design

All analyzes were performed in triplicate. Linear regression analysis pared sample design,an CDA was performed with an analysis of variance (ANOVA) with Duncan's separation of means were carried out, using a probability of 95% ($P<0.05$).

Results and Discussion

Staining Procedure

First stage of the developed protocol.

E. Coli

The Followings Test Were Performed Using This Anaerobic Facultative Bacteria.

Preliminary Test.

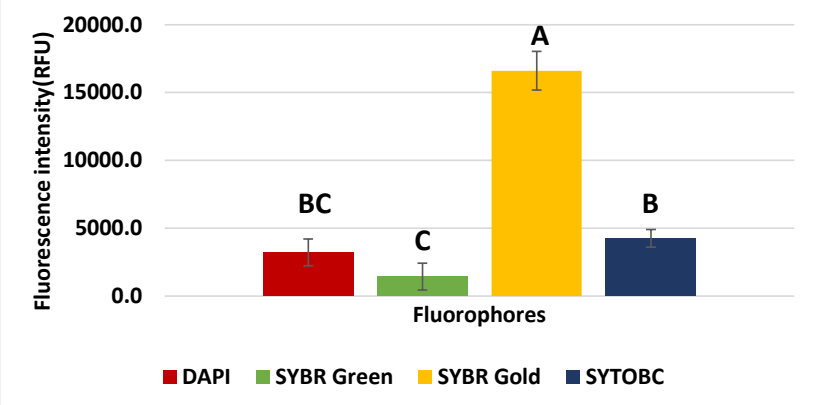
An ANOVA was conducted to compare the fluorescence intensity of four different fluorophores: SYBR Gold, SYBR Green, DAPI, and SYTO BC. The aim was to determine if there were significant differences in fluorescence intensity among these treatments.

Using a significance level set at 0.05, results revealed a statistically significant difference in fluorescence intensity among the evaluated treatments ($F = 0.0001$, $p < 0.05$). Subsequently, a Duncan test was performed as a post hoc analysis to identify specific differences between treatments (Figure 2).

The Duncan test results indicated that the SYBR Gold treatment, with a mean of 16,267.3 RFU, exhibited significant differences compared to the SYBR Green treatment (mean of 3,212.3 RFU), the DAPI treatment (mean of 1,430.0 RFU), and the SYTO BC treatment (mean of 4,245.33 RFU). However, no significant differences were observed between the DAPI and SYTO BC treatments, as well as the SYBR Green and DAPI treatments ($p > 0.05$).

Figure 2

Fluorescence intensity measurements of stained E.coli cells showing comparison between emission of DAPI, SYBR Green, SYBR Gold and SYTO BC.



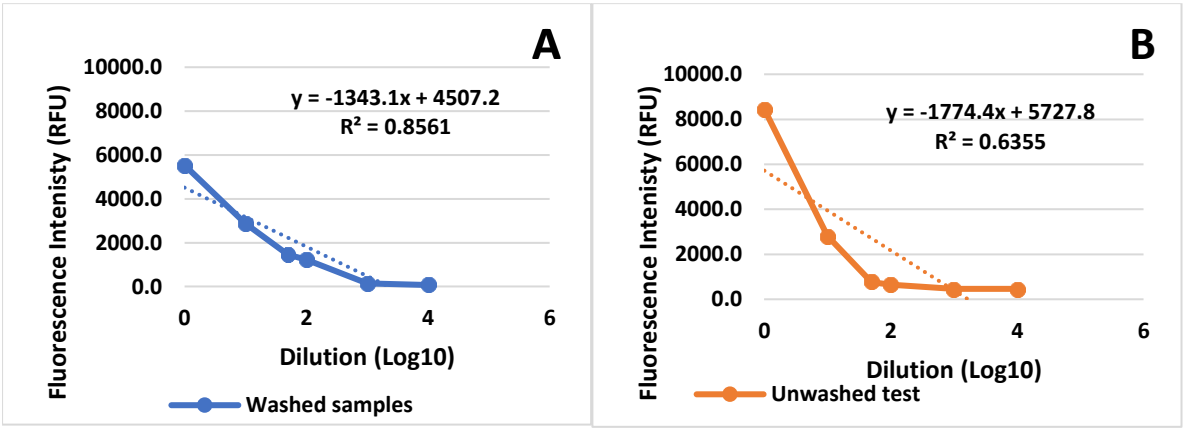
Note. a,b,c: Different letters indicate significant differences ($p<0.05$)

Wash Test

Two sets of samples, one with and one without the wash step, were compared using linear regression analysis; the washed samples presented an R^2 of 0.85 (Fig.3A), being higher than that of the unwashed samples, R^2 of 0.63 (Figure 3**B**).

Figure 3

Fluorescence intensity measurements of stained E. coli cells showing a comparison between staining with a wash step (A) and without a wash step (B)



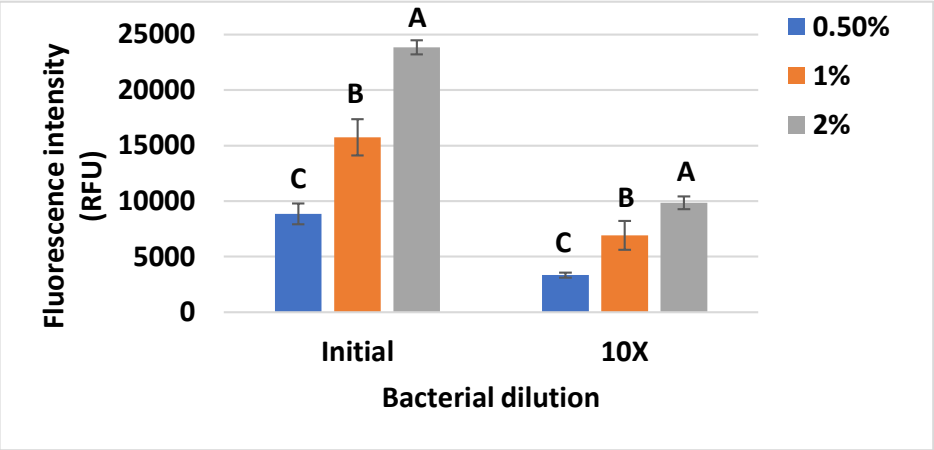
Amount Of Fluorophore.

An ANOVA analysis was carried out to compare the fluorescence intensity between the three concentrations of fluorophore. The results obtained revealed a statistically significant difference in

the concentrations of fluorophore used. After Duncan's multiple range test, it was determined that the treatment with a concentration of 2% SYBR Gold exhibited the highest fluorescence intensity compared to the other treatments tested (Figure 4).

Figure 4

Fluorescence intensity measurements of stained E. coli cells showing a comparison with 3 concentrations of SYBR Gold of the stock solution.



Note. a,b,c: Different upper case letters indicate significant differences ($p<0.05$)

Anaerobic Bacteria

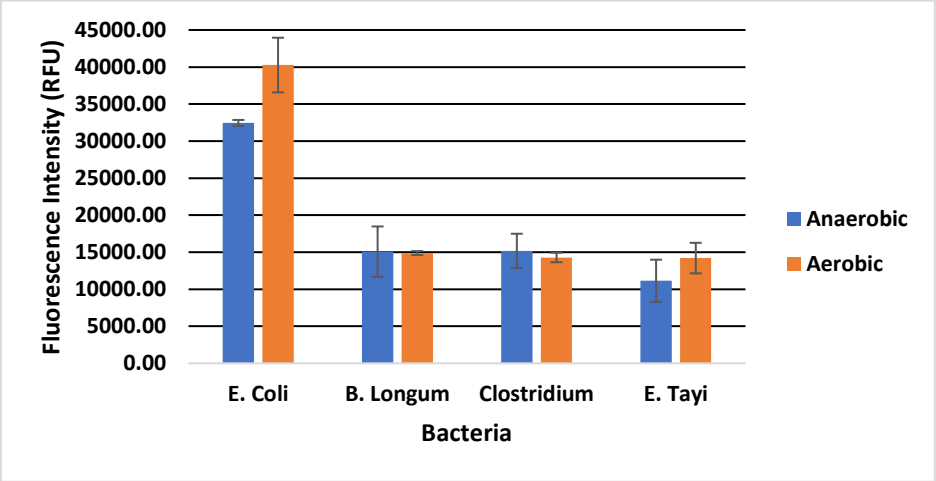
Aerobic Vs Anaerobic Environment

To investigate the suitability of the staining protocol, a test was conducted to determine whether an anaerobic environment was required to effectively stain the bacteria of interest because the preliminary tests were conducted with *E. coli* in an aerobic environment, which may not accurately represent the natural habitat of colonic bacteria known to be anaerobic.

Following the analysis of the results using a pairwise comparison of the samples, it was determined that there was no statistically significant difference in fluorescence intensity between staining the bacteria in aerobic and anaerobic treatments (Figure 5). This suggests that the developed staining method is equally effective in both environments, indicating that an anaerobic setting may not be necessary for achieving satisfactory staining results.

Figure 5

Fluorescence intensity measurements in aerobic environment and anaerobic environment of 3 anaerobic bacteria (B. longum, Clostridium cochlearium and E. tayi) and one stained facultative anaerobic bacterium (E. coli).



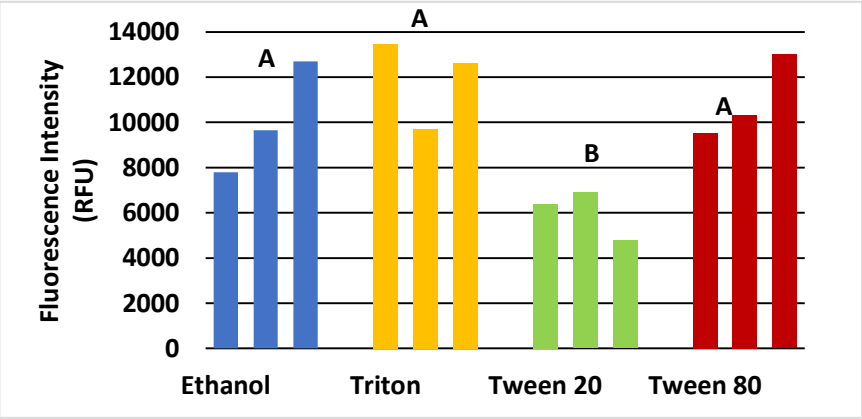
Cell Membrane Permeabilization

To improve the permeabilization of the bacterial cell membrane and enhance the binding of fluorophore to bacterial DNA, an organic solvent and three surfactants, Tween 20, Tween 80, and Triton X100, were individually tested.

It was determined after performing an analysis of variance (ANOVA) and applying the Duncan separation of means test, that there was no significant difference between Triton X100, ethanol, and Tween 80 (falling under category A), while Tween 20 exhibited lower fluorescence intensity (Figure 6).

Figure 6

Fluorescence intensity measurement of the reagents for the permeabilization of the membrane cell.



Note. a,b,c: Different uooer case letters indicate significant differences ($p<0.05$)

Based on these findings, it was decided to continue using ethanol in subsequent analyses due to its accessibility and lower cost (Table 3).

Table 3

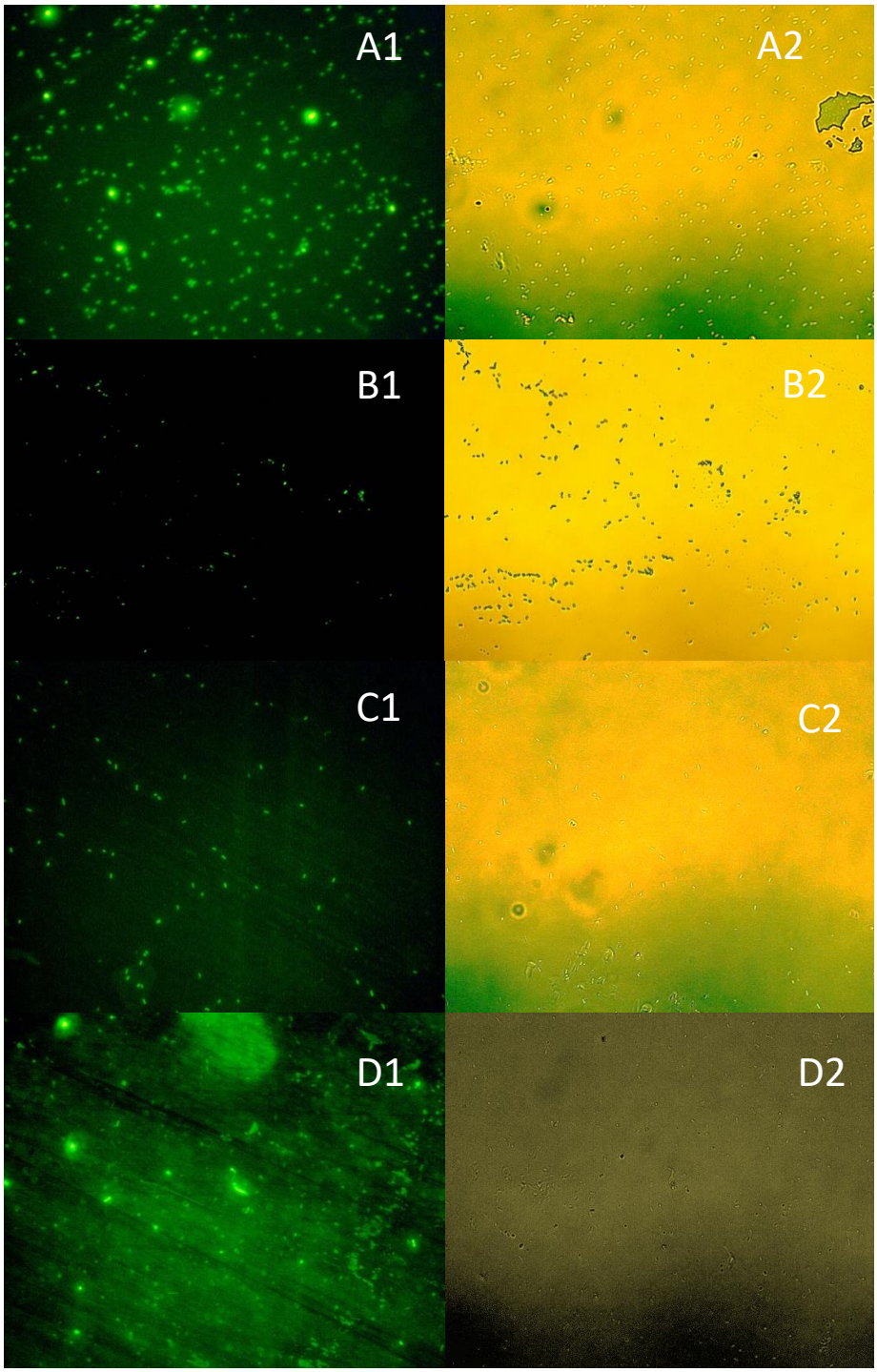
Reagent's prices from Fisher Scientific

Reagents	Price
Ethanol (4 L)	630$
Tween 80 (100 mL)	984$
Tween 20 (100mL)	972$
Triton X100 (250mL)	510.8$

Observations were performed under the microscope on the samples treated with various reagents to conduct a comparative visual evaluation with the optical microscope and using a fluorescence filter cube (Figure 7).

Figure 7

Microscope images of SYBR-gold-stained bacteria obtained using the light mode (2) and fluorescence mode (1). Bacteria were treated with 0.35 M ethanol (A), Tween 80 (B), Tween 20 (C) and Triton X100 (D) before the staining step.



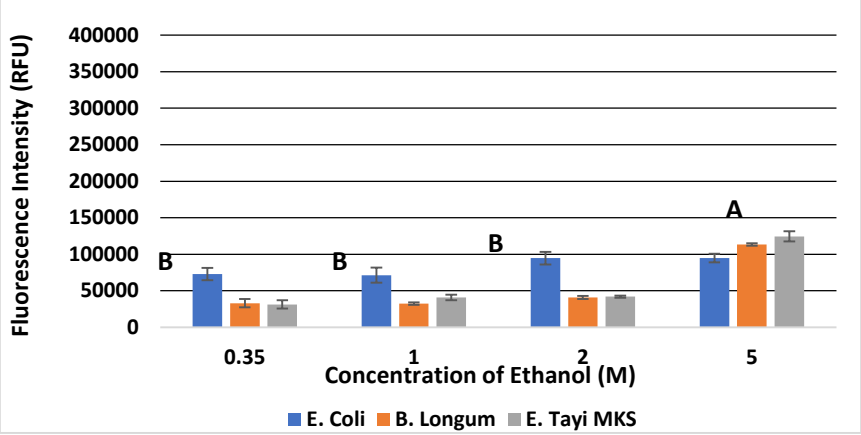
Optimization of Ethanol

Optimization of ethanol concentration to use was performed to determine the optimal point that would allow efficient permeabilization of the cell membrane, thus improving staining and increasing fluorescence intensity. Two experiments were conducted with different ethanol concentrations.

In the first experiment, four ethanol concentrations were tested: 0.35 M, 1 M, 2 M, and 5 M. An analysis of variance (ANOVA) and Duncan's multiple range test were applied. The treatments with 0.35M, 1M, and 2M did not show statistically significant differences but were differentiated from the 5M treatment, which exhibited the highest fluorescence intensity (Figure 8).

Figure 8

Fluorescence intensity measurement with 4 ethanol concentrations: 0.35 M, 1M, 2M and 5M to optimize the cell membrane permeabilization process (First test)

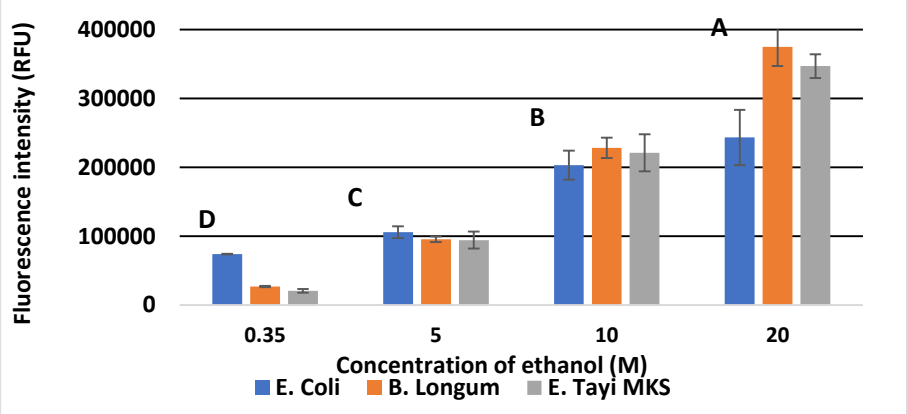


Note. a,b,c: Different letters indicate significant differences ($p<0.05$)

In the second experiment, four ethanol concentrations were tested: 0.35M, 5M, 10M, and 20M. ANOVA and Duncan's test revealed statistically significant differences among all treatments. Among them, the treatment with 5M ethanol concentration showed the highest fluorescence intensity (Figure 9).

Figure 9

Fluorescence intensity measurement with 4 ethanol concentrations: 0.35 M, 5M, 10M and 20M to optimize the cell membrane permeabilization process (Second test).



Note. a,b,c: Different upper case letters indicate significant differences (p<0.05)

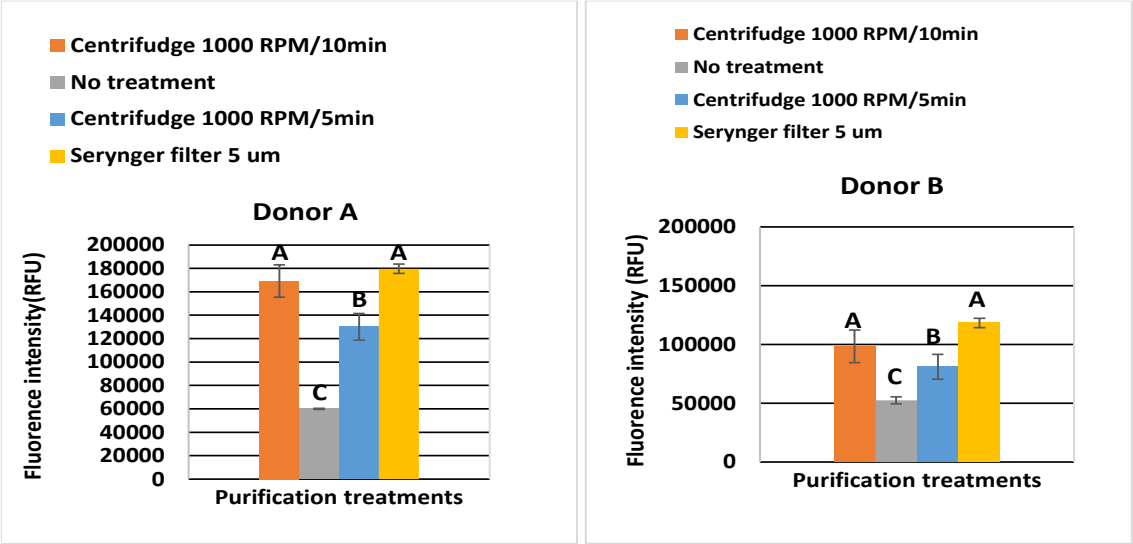
Purification of Colonic Bacteria

An ANOVA was conducted to compare the fluorescence intensity among 4 samples: one sample without treatment (ST) and three with filtration methods: Centrifugation at 1000 RPM for 10 minutes (PF), centrifugation at 1000 RPM for 5 minutes (CN), and syringe filter with a pore size of 5 µm (SY).

The results revealed a significant difference in fluorescence intensity among the evaluated treatments ($p < 0.05$). Duncan test results indicated that centrifugation at 1,000 RPM for 10 minutes and treatment with the syringe filter did not show a statistically significant difference between them. However, both were statistically equivalent to the treatment with centrifugation for 5 minutes. Furthermore, all treatments showed a significant difference compared to the control, as they exhibited significantly higher fluorescence intensity ($p < 0.05$) (Figure 10).

Figure 10

Fluorescence intensity measurements in samples of two donors, A and B, after purification of bacteria using a control (ST) and 3 methods: Centrifugation at 1000 RPM for 10 min (PF), syringe filter with a pore size of 5 µm (SY) and centrifugation at 1,000 RPM for five min (CN)



Note. a,b,c: Different upper case letters indicate significant differences ($p<0.05$)

Estimation of Number of Bacteria

Using the afore mentioned protocol with the respective optimizations, the fluorescence intensity of *E. coli* cells was measured, and cell density was calculated using a hemocytometer (Table 4). The same measurements were carried out for B. longum but over two days (Table 5).

Table 4

Recording of the count of E. coli cells and the corresponding emitted fluorescence intensity over a period of three days.

Day	Fluorescence Intensity (Log10)	Fluorescence Intensity (RFU)	Number of cells	Number of cells (Log10)
1	5.10	124,901.25	925,000,000	8.97
	4.00	10,078.50	92,500,000	7.97
	2.76	582.00	9,250,000	6.97
2	4.98	96,448.00	635,000,000	8.80
	4.06	11,419.67	63,500,000	7.80
	2.84	697.00	6,350,000	6.80
3	5.00	99,514.00	474,000,000	8.68
	3.88	7,668.00	47,400,000	7.68
	2.97	932.00	4,740,000	6.68

Table 5

Recording of the count of B. longum cells and the corresponding emitted fluorescence intensity over a period of two days.

Day	Fluorescence Intensity (Log10)	Fluorescence Intensity (RFU)	Number of cells	Number of cells (Log10)
1	4.76	57419.33	878,333,333	8.94
	4.04	10992.67	87,833,333	7.94
	2.83	671.33	8,783,333	6.94
2	4.73	54216.00	703,333,333	8.85
	4.11	12875.33	70,333,333	7.85
	2.85	711.00	7,033,333	6.85

A statistical correlation analysis was performed between the variables: fluorescence intensity and bacterial count. The results revealed a strong positive correlation, as indicated by a Pearson coefficient of 0.9. Consequently, it can be inferred that there is a predictive relationship between the two variables (Figure 11 and 12).

Figure 11

Relationship between the number of bacteria and fluorescence intensity of stained cells of E. coli

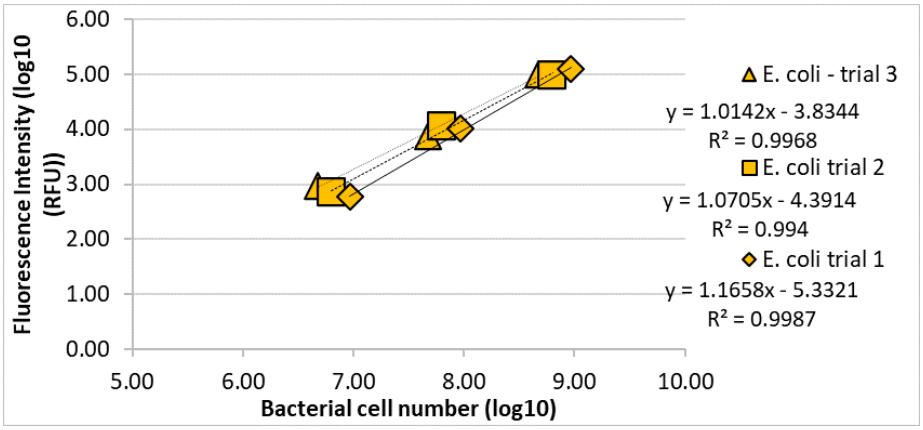
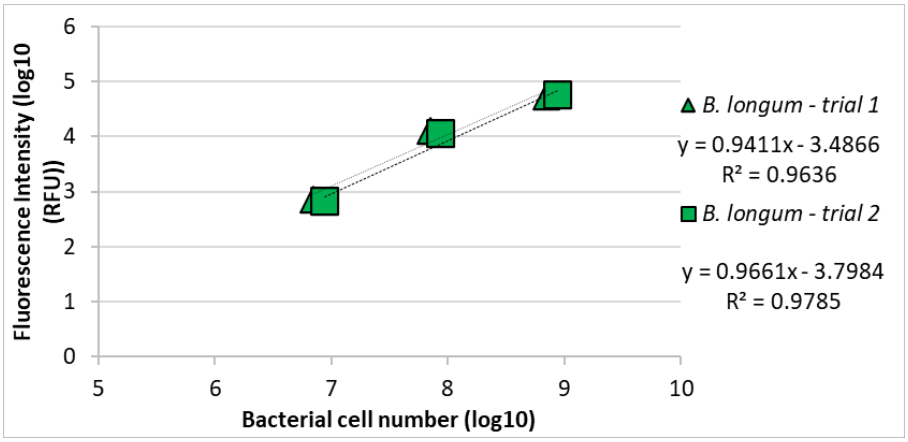


Figure 12

Relationship between the number of bacteria and fluorescence intensity of stained cells of B. longum



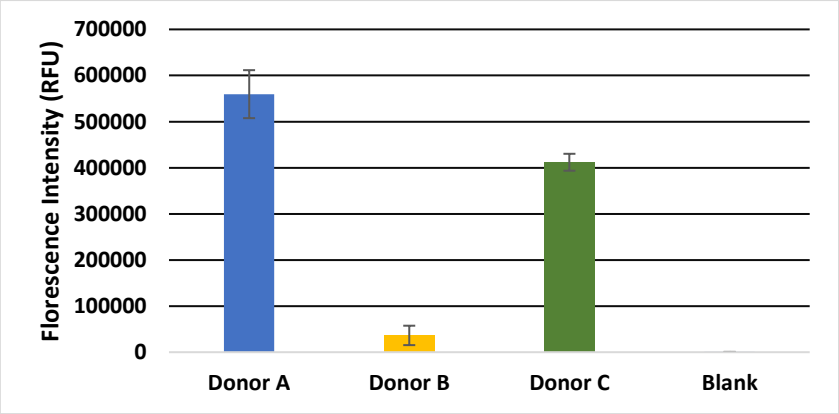
Test of the Protocol

In Vitro Fermentation

The fluorescence intensity of the samples from the 3 donors was measured, and the samples were normalized using the donor sample with the lowest fluorescence intensity (Figure 13).

Figure 13

Fluorescence intensity measurements of samples from 3 donors before normalization and in vitro fermentation, showing differences between donors: D1, D2 and D3.



During the *in-vitro* fermentation the measurement of pH and gas pressure was performed in five timepoints and the distribution of data of pH (Figure 14) and gas pressure (Figure 15) for normalized samples has less variation compared to the distribution of non-normalized samples.

Figure 14

PH values measurements over 48 hours of fermentation showing differences between donors: D1, D2, and D3; and among different substrates: 1) wheat bran, 2) wheat bran arabinoxylan, 3) blank, and 4) positive control.

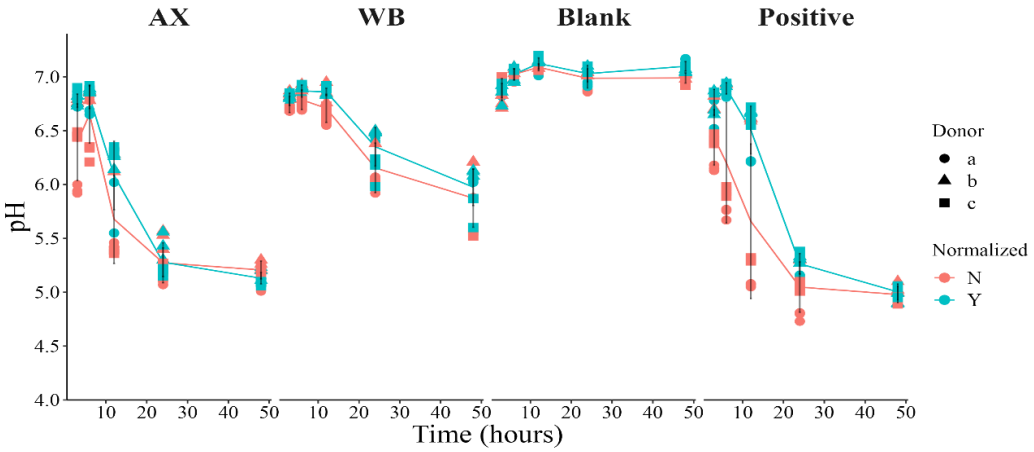
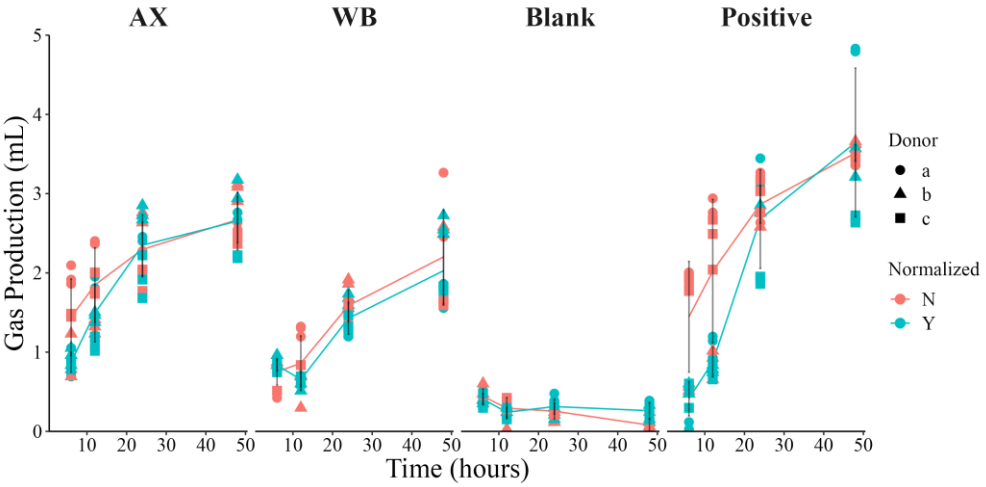


Figure 15

Gas production measurements over 48 hours of fermentation showing differences between donors: D1, D2, and D3; and among different substrates: 1) wheat bran, 2) wheat bran arabinoxylan, 3) blank, and 4) positive control.



Initially, three fluorophores were evaluated: SYBR Gold, SYBR Green, and DAPI, to determine which one exhibited the highest fluorescence intensity in *E. coli* cells. The results obtained indicated that SYBR Gold displayed the highest intensity compared to DAPI and SYBR Green. According to Shibata et al. (2006), both SYBR Gold and SYBR Green should exhibit similar fluorescence intensity, as an exact

1:1 correlation was found for virus and bacteria counts. However, after tests staining marine viruses with both fluorophores, viruses stained with SYBR Gold showed a bright and more stable fluorescent signal compared to SYBR Green I (Noble y Fuhrman, 1998). Considering the 45-minute incubation time of the samples and the subsequent 15-minute washing, the low fluorescence of SYBR Green could be aligned with previous studies that have indicated that the fluorescence of SYBR Green I can fade within 30 seconds under certain conditions, which will result in the use of higher concentrations of SYBR Green I to avoid quenching of the fluorophore (Chen et al., 2001).

DAPI is prone to inaccuracies and has been shown not to be the highest emitting fluorophore (Patel et al., 2007). As mentioned before, SYBR Gold and SYBR Green have high sensitivity for detecting several types of nucleic acids, making them useful for staining both DNA and RNA in bacterial cells. However, DAPI shows significantly higher fluorescence when binding to double-stranded DNA (dsDNA), but its binding capacity to RNA is limited. Additionally, SYBR Green I has also been demonstrated to offer improved staining properties compared to DAPI (Bourzac et al., 2003). Considering that both SYBR Gold and SYBR Green should exhibit similar fluorescence intensity according to Shibata et al. (Shibata et al., 2006), a lower RFU value compared to SYBR Gold can be justified based not only in the superior sensitivity of SYBR Gold but two main factors. First, SYBR Gold has a high fluorescence quantum yield after forming nucleic acid complexes, which is approximately 0.6 to 0.7 (Tuma et al., 1999) being the quantum yield of the DAPI approximately 0.58 (Härd et al., 1990). This means that the complexes formed between SYBR Gold and nucleic acids emit a significantly higher amount of fluorescent light compared to the amount of light absorbed, leading to an increased detectable signal. Second, SYBR Gold undergoes a significant fluorescence enhancement upon binding to nucleic acids, approximately 1000-fold. This enhancement is a direct consequence of the dye's binding to nucleic acids, resulting in a substantial increase in the intensity of emitted fluorescence (Tuma et al., 1999). For this reason, SYBR Gold was chosen as the fluorophore for subsequent analyses due to its superior staining performance.

In other staining protocols, such as crystal violet staining (Ley López et al., 2021) or alcian blue staining to detect biofilm presence (Wu et al., 2020) a washing step is included to remove excess dye. Therefore, the fluorescence was compared using a washing step and one without it. It was observed that the washing step significantly improved the fluorescence intensity, indicating that the samples that were not washed had interference from the remaining fluorophore in the solution. This suggests that the removal of unbound fluorophores contributes to a higher staining efficiency (Figure 4A). This result emphasizes the importance of including a proper washing step in the protocol to enhance staining results.

Additionally, three working concentrations of the fluorophore were tested. Although staining for 30 minutes at 2.5 µM did not show significant differences compared to staining at 5 µM, the reproducibility of the results was better at higher concentration.

In the second stage of the protocol development a decrease in fluorescence intensity was observed during the staining of anaerobic bacteria. To improve the staining of gram positive bacteria, experiments were conducted using three detergents (Triton X-100, Tween-20, Tween 80) and an organic solvent (ethanol) to enhance permeability and improve staining of bacterial cells. Organic solvents are capable of dissolving cell membrane lipids, thereby increasing membrane permeability (Jamur y Oliver, 2010). Detergents, on the other hand, are widely used in biology as membrane permeabilizing agents (Koley y Bard, 2010).

Therefore, TritonX-100, one of the most widely used nonionic surfactants to permeabilize living cell membrane, and Tween-20, a nonionic detergent that can solubilize cell membrane without affecting cell membrane integrity, were chosen. They can create pores large enough for oligonucleotide probe to go through without dissolving the plasma membrane. They are suitable for antigens and nucleic acids in the cytoplasm (Amidzadeh et al., 2014). The results of the treatments showed positive outcomes, indicating an improvement in fluorescence intensity. These results are consistent with previous studies (Amirilargani et al., 2009; Johnson, 2013; Le Maire et al., 2000).

Therefore, ethanol was chosen to be used for the remaining experiments due to its availability and lower cost.

Knowing that gram-positive bacteria survive and grow in environments with higher ethanol content, we proceeded to optimize the amount of this to improve cell membrane permeability (Liu y Qureshi, 2009). Ethanol has a pronounced effect on the structural properties of membranes, making lipid bilayers more fluid and permeable (Patra et al., 2006). Its presence disrupts the hydrocarbon chains of lipids, resulting in an increase in lipid area and overall membrane fluidity (Gurtovenko y Anwar, 2009). This facilitates the penetration of the fluorophore into the cells. Additionally, ethanol has the capacity to not damage nucleic acids (Marquina et al., 2021). This could explain the high fluorescence intensity of stained bacterial cells observed after adding 20 M ethanol.

To eliminate any residue that may interfere with the staining of bacteria in fecal samples, three purification methods were compared with the control: two centrifugation times at low speed and a 5 µm syringe filter. The size of the syringe filter was selected considering that the size of the bacteria ranges between 0.5 and 3 µm, with the potential to reach 10 µm and considering that the medically relevant bacteria of interest have a size between 0.4 and 2 µm (Pírez y Mota, 2006).

All treatments showed a significant difference compared to the control (Figure 10). The treatments using the syringe filter and the higher centrifugation speed resulted in significantly higher fluorescence intensity.

To assess the protocol's efficacy, an in-vitro batch fermentation was conducted using colonic bacteria sourced from three distinct donors. This fermentation procedure encompassed two distinct cohorts: the initial cohort constituted unnormalized samples, while the subsequent group comprised normalized samples. Monitoring both the control and normalized samples, pH levels (Figure 7) and gas pressure (Figure 8) measurements were performed.

Minimal deviations were observed initially in the first gas pressure and pH measurements, underscoring the impact of the normalization process (Yao et al., 2022; Yao et al., 2023). Prior research has indicated significant discrepancies in pH and gas pressure readings across various donors. Gas

production exhibited an observable post-normalization effect. Normalized samples commenced at comparable levels, subsequently diverging, and manifesting varied responses after the 24- or 48-hour intervals. In contrast, control samples exhibited a distinct pattern: although they commenced at varying levels, after 48 hours, all samples converged towards a common point.

Conclusions

A method was developed to measure bacterial cell load in depleted media (after fermentation) using a fluorophore. For the staining procedure, SYBR Gold was chosen as the best fluorophore and a stock solution of 2% is enough to stain bacterial cells without difficulty in an aerobic environment. Regarding the cell permeabilization process, it was found that ethanol was the best agent used, and it was determined that an amount of 15 µL of ethanol was the most effective. The method for monocultures of bacteria was optimized. In addition, this method was used to normalize the bacterial cell load of diluted fecal samples, finding that the best method to purify bacteria from the samples to stain them was centrifugation at 1000RPM for 10 min.

The fluorescence intensity could not be correlated with the number of bacterial cells in the fecal samples because there may be variations by genome size and variation in the composition of the microbiome.

In the fermentation experiment, there were an equal pH and gas pressure values in the first measurements which may indicate a positive effect of normalizing the bacterial load using the developed protocol. These observation in changes of pH and gas pressure were observed in all the substrates studied.

Recommendations

Use a 10 µm syringe filter after centrifugation in the purification step, to obtain a more purified sample of bacteria.

Perform correlations of fluorescence intensity and number of bacteria with isolated bacteria from fecal samples to determine if the effect of genome size has a significant impact.

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