University of Reading

# γ-Aminobutyric Acid Production by Lactic Acid Bacteria in the Gut Microbiota

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Date: March 2019

**Declaration**

I declare that this thesis is my own work and the contribution of others has been properly and fully acknowledged.

Adebambo Oluwabunmi Ikeoluwa, 2019

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## Acknowledgement

I will like to give all thanks and glory to the almighty God without whom I am nothing for the grace and strength to finish this race. I will like to thank my supervisor Dr Kimon-Andreas Karatzas for his continuous support and guidance during my PhD. I will like to thank every member of my research group for their support and assistance at every point of my research work not forgetting Holly Wang especially who has played a massive role in me achieving this success, I cannot thank you enough. I will also like to thank members of 3.18 laboratory as well as the lab technicians your help at any point is much appreciated.

Special thanks goes to my dad, Late Prince (Dr) Victor Adebambo although you are not here with us I am sure you are watching down from heaven with a big smile on your face and to my wonderful and ever caring mum, Prof (Mrs) Oluwafunmilayo Adebambo for sponsoring me through this journey. You believed so much in me even when I was about giving up and you were ready to spend all you had to give me this quality education. You are a true mother and my role model. I cannot thank you enough because you made me who I am today. I love you so much and you know that. To my siblings, In-laws, nephews and nieces thanks for being part of my success story.

Special appreciation goes to my darling husband Mr Igwe Emmanuel Obaji and my beautiful daughters Miss Shalom Oluwamomi and Sharon Oluebube Obaji without you all I am incomplete. Thanks for your continuous love and support and for also accepting me with my short comings. I love you loads and forever will.

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**Chapter 4: Modulation of the gut microflora γ-aminobutyric (GABA) and short chain fatty acids (SCFAs) production through feed in pH-controlled single stage continuous culture fermentation and static batch culture fermentation**

# General abstract

γ-aminobutyric acid (GABA) is a non-protein amino acid which functions as an inhibitory neurotransmitter of the mammalian brain. orally administered GABA has tranquilizing and diuretic properties, and also relieves depression, anxiety, and most importantly reduce blood pressure. Due to its beneficial physiological functions research has focused on identification and isolation of bacterial strains producing high levels of GABA, which could be used in the production of fermented food products. This process is costly and labour intensive therefore, we developed a rapid colorimetric pre-screening test which could reduce costs in the isolation of GABA producers from various environments.

Lactic acid bacteria (LAB) are the most important group of probiotics and starter cultures. Although fermented products are known sources of LAB, the human gut microbiota is also considered a good source as its natural habitat. However, isolates to be classified as probiotic, should be safe, withstand processes and exert a beneficial effect on the host. Therefore, various *in-vitro* tests were carried out to determine probiotic potential of isolated GABA producing strains. *Lactobacillus plantarum strains* showed similar characteristics with the reference strain *Lactobacillus casei shirota* which is a widely used probiotic.

To increase the GABA levels in the gut of the host. high GABA producers could be delivered to the host through fermented products or apply dietary interventions that could increase GABA levels in the gut. We decided to investigate the 2nd strategy by using single stage, batch culture systems simulating the distal colon and also three-stage continuous colonic model system simulating the whole colon with inoculum from healthy donor. We focused on supplementing these systems with amino acids and peptides other than glutamate and look at a possible increase in GABA and SCFA levels.

We found that amino acids (casamino acids, L-cysteine e.t.c), other than glutamate are able to increase gut microbiota mediated GABA and SCFA production in the colon. This suggests that increased consumption of protein –based food or dietary protein in our diets could possibly increase the production of GABA and SCFAs in the gut.

# Abbreviations

| | |
|---|---|
| BHI | brain heart infusion |
| Bp | base pair |
| °C | Degree Celsius |
| CFU | colony forming unit |
| CNS | central nervous system |
| DNA | Deoxyribonucleic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EFSA | European Food Safety Authority |
| FAO | Food and Agriculture Organization of the United Nations |
| GABA | gamma aminobutyric acid |
| GAD | glutamate decarboxylase |
| GC | gas chromatography |
| GCMS | Gas Chromatography and Mass spectrometry |
| GIT | gastrointestinal tract |
| GRAS | generally regarded as safe |
| h | hours |
| $H_2O$ | water |
| HCl | hydrochloric acid |

| | |
|---|---|
| HPLC | high-pressure liquid chromatography |
| IBD | Inflammatory Bowel Disease |
| IBS | Irritable Bowel Syndrome |
| l | litre |
| LAB | lactic acid producing bacteria |
| MIC | minimum inhibitory concentration |
| MRS | De Man Rogosa and Sharp |
| M | molar |
| mg | milli grams |
| MHz | mega hertz |
| Min | minutes |
| mL | milli litre |
| mm | milli metre |
| mM | milli molar |
| Na+ | sodium |
| NaCl | sodium chloride |
| NaOH | sodium hydroxide |
| OTU | operational taxonomic unit |
| PBS | phosphate buffer solution |
| PCR | polymerase chain reaction |

PLP pyriodoxal -5- phosphate

pH potential of hydrogen

RDA recommended dietary allowance

RNA ribonucleic acid

s second

SCFA short chain fatty acid

SD standard deviation

SS steady state

Tris/HCl Tris hydrochloride

U unit

V Vessel

Vit vitamins

WHO World Health Organization

µg micro gram

µL micro litre

µm micro metre

% percent

# Chapter 1

# Literature Review

## 1. Introduction

The gut microbiota is essential for human health and plays a major role in the activation of the immune system and the central nervous system i.e. there is a connection between the gut and the brain and its study is a hot topic especially in neuroscience, as research in this field has shown that the development of the brain system is dependent on gut microbiota (Evrensel and Ceylan, 2015). Gut microorganisms are able to produce and deliver neuroactive substances such as γ-aminobutyric acid (GABA) that acts on the gut-brain axis which is a two-way pathway of communication between the gastrointestinal tract (GIT) and the central nervous system (CNS). Studies have shown the ability of the gut microbiota to regulate the gut and brain function for example, its effects on improving mood, behaviour as well as human cognitive function (Mazzoli and Pessione, 2016). However, production of GABA from different plant sources has yielded much low amount of GABA concentration and thus has led to the study of production of GABA via fermentation by using microorganisms such as lactic acid bacteria which are considered to be more efficient and more convenient to use in food processing and production (Lu et al., 2008). Microbes that have a glutamate decarboxylase system such as lactic acid bacteria, yeast, fungi and other microorganisms have been studied extensively for the production of GABA (Feehily et al., 2013, Dover and Halpern, 1972, Karatzas et al., 2010). Of these microbes, LAB have been of utmost importance for the production of GABA in the food industry as they are "generally recognised as safe" (GRAS) and they play an important role in fermentation especially in the production of dairy products due to their functional and probiotic

properties (Ali, 2010). Given the positive health effects GABA has been shown to elicit, GABA production could also be considered a probiotic property.

GABA is a four carbon non-protein amino acid that functions majorly as an inhibitory neurotransmitter of the mammalian brain (Hudec et al., 2014). Studies have shown that orally administered GABA has tranquilizing and diuretic properties, as well as an ability to relieve depression, anxiety, sleeping disorders and most importantly a hypotensive effect (Siragusa et al., 2007, Distler et al., 2012, Tujioka et al., 2009, Padgett et al., 2012). Due to these important physiological effects, the production of GABA enriched functional foods has been on the increase and this has led scientists studying the bacterial glutamate decarboxylase system (GAD) that is responsible for the production of GABA. The GAD system is a very powerful mechanism of acid resistance in many microorganisms including LAB, and it functions with the help of the GAD enzymes (GadD1, GadD2) which work together with antiporters (GadT1, GadT2) and play a major role during acidic stress by allowing the growth and survival of bacteria under mildly and severe acidic conditions (Cotter et al., 2001b). The GAD system has a very high output and as such is an important acid resistance system (Feehily and Karatzas, 2013).

### 1.1 Lactic acid bacteria (LAB): Taxonomy and Characteristics

The concept and development of LAB can be dated back to the early 1900s. Since then, much attention has been given by scientists to the study of lactic acid bacteria and their application in food (Stiles and Holzapfel, 1997). LAB is ubiquitous and can be found mainly in carbohydrate rich environments. They are also part of the normal microbiota present in human and animal bodies (Florou-Paneri et al., 2013).

Phylogenetically, LAB are Gram positive, non-spore forming, facultative anaerobic, acid tolerant and catalase negative bacteria. Morphologically, they can be divided into rods and

cocci, while they can also be divided according to their major metabolic fermentation end-product which can either be a mixture of lactic acid, carbon dioxide, acetic acid/ethanol (hetero-fermentative) or only lactic acid (homo-fermentative); (Florou-Paneri et al., 2013, Ali, 2010).

Due to the role lactic acid bacteria play during fermentation and because of their GRAS status "Generally Regarded as Safe" they have been of utmost importance in the food/feed industry, medical, pharmaceutical and chemical industries. LAB have been used majorly as a functional ingredient in the production of enzymes, low calorie sweeteners, starter culture during fermentation, antimicrobial agents, exopolysaccharides, and also function as vaccine delivery vehicles.

### 1.2 Glutamate Decarboxylase (GAD) systems

Glutamate is a non-essential amino acid that has been known to play a major role in a big range of metabolic processes such as glycolysis and protein synthesis (Cotter and Hill, 2003). The GAD system is involved in various stress responses with regards to animals and plants but in microorganisms, it plays a major role in acid resistance i.e. survival at low pH. GAD enzyme is pyridoxal-5-phosphate dependent and catalyses the irreversible decarboxylation of L-glutamate to GABA with the consumption of protons, thus increasing the intracellular pH. (Feehily and Karatzas, 2013). At low pH, the GAD system imports extracellular glutamate with the help of an antiporter in exchange for an extracellular GABA by consuming protons i.e. L-Glu + $H^+$ $\xrightarrow{GAD}$ GABA + $CO_2$ (Karatzas et al., 2012).

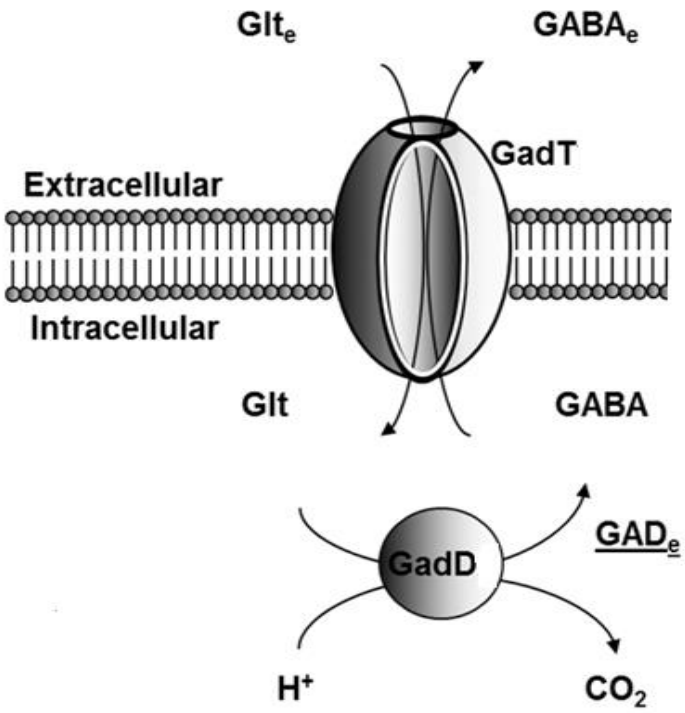


**Fig 1:** Depiction of the model for the function of the GAD system. The GadT antiporter imports extracellular Glt, which is decarboxylated by GadD to GABA, proton is consumed during this process (H+*). GABA is then exported by the GadT with the simultaneous import of Glt. (Karatzas et al. 2012).

The glutamate /GABA antiporter as well as the cytoplasmic glutamate decarboxylase are the two major proteins that help in the conversion of glutamate to GABA and $CO_2$ by consuming protons (Cotter et al., 2001a). The removal of protons during the decarboxylation reaction helps in protecting the cells against acidic environments by preventing excess acid from getting into the cytoplasm (Karatzas et al., 2010).

The GAD system is present in GABA producing LAB genus for example *Lactobacillus* and *Lactococcus* and it is essential as an acid resistance mechanism needed in maintaining the viability of the microorganisms under acidic conditions during anaerobic fermentation process (Komatsuzaki et al., 2005, Nomura et al., 1999, Siragusa et al., 2007).

### 1.3 γ-aminobutyric acid (GABA): Relationship with the gut-brain axis

The gut brain axis is useful in the treatment of neuropsychiatric disorders and the gastrointestinal system is known to be the largest organ (Evrensel and Ceylan, 2015). Research has shown that the gut microbiota influences our mood and plays a major role in neurological development and behaviour (Foster and McVey Neufeld, 2013, Crumeyrolle-Arias et al., 2014, Bravo et al., 2012).

The gut microbiota produces neuroactive metabolites such as GABA. These metabolites as well as the vagus nerve could be the link between the gut and brain without excluding other possibilities (Mayer et al., Holzer and Farzi, 2014). GABA is the main inhibitory neurotransmitter in the central nervous system of mammals and acts by binding to either the $GABA_A$ or $GABA_B$ receptors (Li et al., 2010, Dhakal et al., 2012b, Wang and Kriegstein, 2009). It is a non-protein amino acid that is widely distributed in nature i.e. it is found in plants, mammals and some insects and it affects the Krebs cycle since GABA is metabolised by transamination with α-ketoglutarate to produce glutamate and succinic semialdehyde which is then oxidised to succinate that feeds into the Krebs cycle. (Diana et al., 2014). The factors that affect GABA production are pH, temperature, culture time and media additives such as glutamate (Dhakal et al., 2012b). The $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic) receptors are responsible for the alterations in the GABAergic system also play an important role in stress-related psychiatric conditions (Bravo et al., 2011). Therefore, the gut microbiota can be regulated through the use of probiotics, changes in diet or faecal microbial transplantation. Therefore, modulating the gut microbiota could possibly help in dealing with various neuropsychiatric disorders.

### 1.3.1 Physiological functions of GABA

GABA can be found in neural and non-neural tissues outside the brain including the gastrointestinal tract and it participates in regulating various neural signal pathways and physiological functions which have been widely studied and these studies have led to the development of GABA-rich functional foods. It has majorly been reported to regulate blood pressure in both hypertensive rats and human intervention trials (Hayakawa et al., 2004, Inoue et al., 2003, Tanaka et al., 2009). Hypertension is a major cardiovascular disease, therefore a reduction in the high blood pressure will also help in controlling other related diseases and illnesses thus, production of GABA and GABA-rich functional food could have a massive health benefit for patients suffering from cardiovascular diseases and other related illnesses. A variation i.e. increase or decrease in GABA production has been linked with various diseases such as Parkinson's, seizures, Alzheimer's as the GAD substrate L-glutamate stimulates and acts as a neuro-inhibitor (Ting Wong et al., 2003, Battaglioli et al., 2003). GABA has also been found to help in the prevention of sleeping disorders, mood disorders, depression and it improves immunity against stress and alcohol- related disorders as it protects against kidney diseases while activating the functions of the kidneys and liver (Ting Wong et al., 2003, Sasaki et al., 2006, Krystal et al., 2002, Bjork et al., 2001). It also prevents against diabetes as it has been proven that GABA helps in insulin secretion (Adeghate and Ponery, 2002). GABA has also been shown to slow down the proliferation of various cancer cells while it is also considered to help in suppressing tumours (Schuller et al., 2008, Oh and Oh, 2004, Kleinrok et al., 1998). Hormone secretion could be regulated by GABA, hence it has been shown to increase growth hormone, improve the secretion of insulin and also improve the function of the thyroid hormone (Adeghate and Ponery, 2002, Wiens and Trudeau, 2006, Xie et al., 2014).

Some reasons of GABA being effective in terms of its major health benefits could possibly be due to its relaxing effect on users by inhibiting the nerve cells in the brain from receiving much

stimulatory effects which in turn cause the patients to maintain a relatively calm and relaxed state (Harris and Allan, 1985). The GABA receptors ($GABA_A$ and $GABA_B$) have been implicated in the various physiological activities of GABA for example GABA functions by binding to its specific receptors and it activates the receptors to regulate the chloride ion distribution between the nerve cell membranes i.e. they increase membrane conductance of chloride ion in the nerve cells. The activated GABA receptors ($GABA_A$ and $GABA_B$) causes an influx of chloride ion into the nerve cells by opening the chlorine ion channels and thus causing hyperpolarisation or reducing neural excitability in the cell membrane which in turn results in GABA secreting excess salt to be able to lower blood pressure or reduce the activity of the cell (Harris and Allan, 1985, Ma et al., 2015). Also, studies have shown that GABA receptors are expressed and activated in tumour cells and also GABA might be involved in the migration of tumour cells in a way that is similar to that of chemokines as GABA through the GABA receptors affects every stage of cell development such as proliferation, migration and differentiation therefore levels of GABA receptors are upregulated in cancer cells thus raising the possibility of reducing the activities of the cells by manipulating the GABA receptors. (Joseph et al., 2002, Ortega, 2003, Takehara et al., 2007, Watanabe et al., 2006).

### 1.3.2 Factors affecting GABA production

Various factors have been implicated in the production of GABA and these factors include temperature, pH, fermentation time and media additives such as glutamate. These conditions can be optimised based on the biochemical characteristics of the microorganisms during fermentation. Therefore, the ability to produce GABA / respond to changes in acidic condition differs in different species of microorganisms, and this could be as a result in the difference in the GAD system of each microorganism (Dhakal et al., 2012a, Tajabadi et al., 2015). GABA synthesis usually depends on pH changes during the fermentation process, these pH changes are dependent on the species of microorganisms, the fermentation time and fermentation

temperature. Hence, GABA production depends on fermentation temperature and time. Studies have shown that optimal GABA production was achieved 48 h and at temperatures ranging from 25 $^{O}$C to 40 $^{O}$C. (Dhakal et al., 2012b, Li et al., 2009). Likewise, the nutrient composition such as carbon and nitrogen sources and media additives such as glutamate and PLP co-enzymes of the GAD system, are also factors that affect the production of GABA during the fermentation process (Cho et al., 2007).

### 1.4 Gut microbiota

Foods consumed by humans travels through the gastrointestinal tract (GIT) and with these foods, microorganisms are also transported to the stomach where digestive enzymes such as pepsin are released, hydrochloric acid is also released which in turn lowers the pH in the stomach. The food then passes through the small intestine, duodenum, jejunum and onto the ileum, during this process the pH increases from 6.6-7.5 and more digestive enzymes are released which leads to cleavage and absorption of carbohydrates, proteins and lipids. Then finally the luminal; content is passed to the colon (pH 5.4 - 6.9) which is divided into ascending, transcending, descending and sigmoidal colon where water and salts are absorbed and the remaining luminal contents are passed out of the body as faeces (Evans et al., 1988, Sekirov et al., 2010, Roeselers et al., 2012, Macfarlane and Macfarlane, 2007a).

The human GIT is generally an anaerobic environment but conditions such as transit time, pH, nutrient availability and substrate changes affect the microbial ecosystem. This lead to an accumulation of microorganisms that make up the complex microbial ecosystem known as the gut microbiota consisting of bacteria, yeast, fungi, archaea and viruses. This community makes up to $10^{13}$ and $10^{14}$ microorganisms that dominate the gut (Sekirov et al., 2010). The composition of the gut microbiota differs as concentrations of bacteria increase from the stomach to the colon; with $10^{3}$ colony forming unit (CFU) of live bacterial cells per ml of

sample in the stomach and duodenum to $10^4$ -$10^8$ in the jejunum and $10^9$-$10^{12}$ in the colon (Blaut and Clavel, 2007). This complex ecosystem is represented by fewer bacterial phyla which are *Firmicutes, Bacteriodetes, Proteobacteria, Fusobacteria, Verrucomicrobia* and *Acticobacteria.* The intestinal microbiota of newborn is dominated by *Proteobacteria* and *Actinobacteria* which then after some time becomes dominated by *Firmicutes* and *Bacteriodetes* which are dominant representatives of the adult intestinal tract. The main genera present in the *Firmicutes* phyla are the *Feacalibacterium, Lactobacillus* and *Roseburia* while the *Bacteroides, Alistipes* and *Prevotella* are present in the *Bacteriodetes* phyla. *Bifidobacterium* and *Collinsella* are also the genera present in the gut microbiota under the *Actinobacterium* phyla (Quigley, 2013, Eckburg et al., 2005).

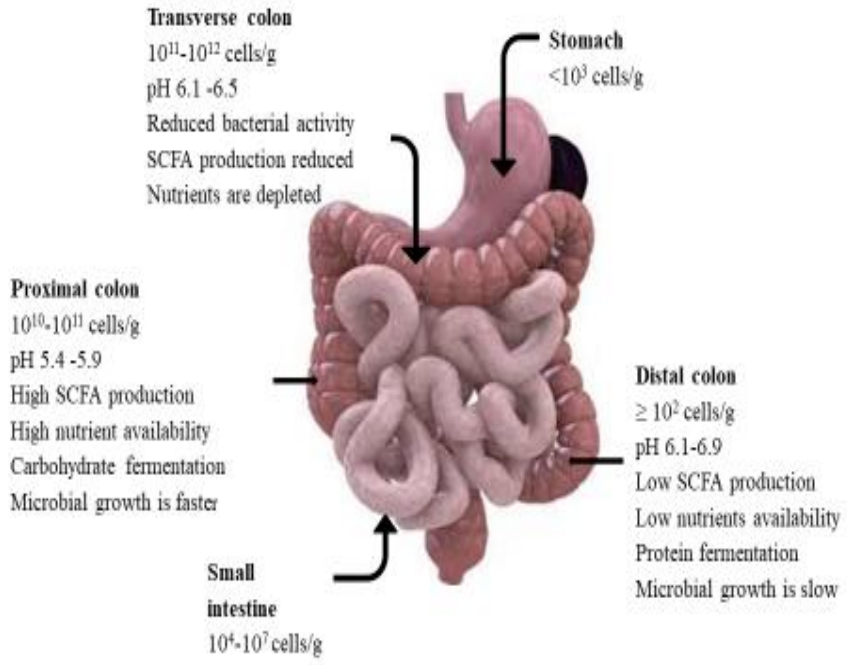


**Fig 2:** Diagram showing the different segments of the human gastro intestinal tract: nutrient availability, bacterial cells/grams, bacterial fermentation activity (Payne et al., 2012).

### 1.4.1 Importance of gut microbiota

The gut microbiota has a symbiotic and commensal relationship with its host and they are present in the gut due to the utilization of available substrates used up during saccharolytic or proteolytic fermentation (Bäckhed et al., 2005). This commensal relationship has been widely studied to determine the importance of the intestinal microbiota on the host cell. From these studies, the gut microbiota has been useful in promoting homeostatic functions like immunomodulation, maintaining the intestinal mucosa, upregulation of cytoprotective genes, regulation of apoptosis (Patel and Lin, 2010, Lin et al., 2008).

They also perform a protective role against pathogenic bacteria trying to colonise the gut by competing for available nutrients, space, receptors and also producing substances such as fatty acids, peroxides, bacteriocins which could inhibit the growth of pathogens while some produce proteases that help in denaturing the toxins produced(Castagliuolo et al., 1999, Quigley, 2013). Apart from the protective roles the intestinal microbiota play, they also have some metabolic potential such as the production of vitamins (such as folate and Vit K), short chain fatty acids that promotes the growth of the intestinal epithelial cells, and also chemicals which are neurotransmitters and neuromodulators for example GABA (Quigley, 2013, Nicholson et al., 2012).

### 1.4.2 Factors affecting gut colonisation

There are different studies carried out to show or analyse the bacteria colonising the gut of the new-born infants and these studies have shown the dominant bacterial population as *Enterococcus* and *Streptococcus* with *E. feacalis* and *S. salivarus* as the most abundant (Tremaroli and Backhed, 2012).

New-born infants are born with underdeveloped GIT as they develop in the chorion which is a germ-free environment. Once the infant leaves the womb/chorion and enters into a more

complex environment during birth, they get colonised by microorganisms acquired from the mother and also from the environment before a more stable gut microbiota develops after weaning, through life until adulthood (Roeselers et al., 2012). There are a number of endogenic factors (such as sex, gestational age, genetics and immunity) and external factors (such as mode of delivery, mode of feeding i.e. breastfed or formula fed, diet, antibiotics and environment) that can affect the human gut microbiota composition (O'Toole and Claesson, 2010, Roeselers et al., 2012, Qin et al., 2010).

### 1.4.3 Gut microbiota and diseases

There are two major mechanisms that control the composition of the gut microbiota and theses are host-bacteria interactions which are basically the immunological interactions that occur in the gut and also bacterial-bacteria interactions which are changes caused by the production of metabolites, consumption of oxygen, and nutrient competition (Schiffrin and Blum, 2002, Roeselers et al., 2012). Therefore, changes caused by these interactions can also lead to a shift in the bacterial population colonising the gut and this can in turn lead to dysbiosis in the gut.

Dysbiosis results in an increase in pathogenic bacteria in the gut and this may affect the health of the host. Increase in incidence of dysbiosis has raised the interest of researchers in controlling the functions and composition of gut microbiome to benefit the host health(Nicholson et al., 2012). However, the physiological state of the host has been linked to the diversity and composition of the gut microbiota for instance, studies have shown a lower ratio of *Bacteriodetes* to *Firmicutes* in obese subjects compared to their normal weight counterparts (Ley et al., 2006, Turnbaugh et al., 2006). Several diseases such as irritable bowel syndrome (IBS), Alzheimer's, Crohn's, depression, anxiety, autism etc. have been associated with dysbiosis in the gut and various novel therapeutic tools such as antibiotics, probiotics,

prebiotics, synbiotics (probiotics + prebiotics), faecal transplantation /bacteriotherapy have been used to regulate the gut microbiota (Petrof et al., 2013, Lee et al., 2016, de Vos and de Vos, 2012, Bäckhed et al., 2012).

### 1.5 In vitro gut fermentation.

The human gut which is host to a complex microbial community can be widely studied using healthy individuals, hospital patients or sudden death victims. Therefore, direct study of the human gut using *in-vivo* models has its biological significance as well as its setbacks because there are a wide range of factors that may affect the gut microbiota which are difficult to control in human studies (Macfarlane and Macfarlane, 2007a, Dave et al., 2012). For instance, research has been able to show that there is a variation in the human gut microbiota and also that individuals react differently to treatments they are subjected to which could be due to individual genetic makeup. These variations make it difficult to control or correlate the results obtained from these treatments and this in turn makes it difficult to compare gut microbiota changes to the treatment of interest (Dave et al., 2012, Dethlefsen and Relman, 2011).

In addition, ethical considerations and approvals can limit the use of human studies to obtaining and studying faecal samples while human studies are generally expensive, time consuming and require specialist facilities. These can lead to difficulty in getting the right number of volunteers for the study while volunteers tend to often drop out from the study or become non-compliant (Macfarlane and Macfarlane, 2007a). The technical and ethical problems encountered while using human subjects often leads to the use of animal and *in-vitro* models to increase our understanding of the gut and the gut microbiota. In animal models, the researcher is able to carry out more controlled and reproducible experiments while being able minimise the changes that occur due to environmental and genetically variability (Antonopoulos et al., 2009). Also, another advantage of using animal models is that they provide direct access to the intestinal

contents, tissues and the researcher has more liberty and flexibility in trying to test for drugs or toxins (Macfarlane and Macfarlane, 2007a). However, concerns have been raised about the use of animal models as there are significant differences in digestive physiology of human and the animal model used. For example, the digestive tracts of pigs is similar to that of human than that of rats except that the proximal colon of pigs is densely populated and these differences raises concern with relevance to the data obtained from these studies (Payne et al., 2012). Using *in-vivo* models such as human and animal models is generally expensive and requires the use of specialist facilities. Therefore, the use of *in-vitro* models to study the gut microbiota outweighs the limitations and set back of using *in-vivo* models.

*In-vitro* human gut models are mainly used to culture stable but complex intestinal microbial population in a more controlled environment, These *in-vitro* models (batch or continuous) comprise single or multiple vessels that contain growth medium that simulates the gut which is inoculated with faeces under anaerobic conditions, controlled pH and temperature (Payne et al., 2012, Eckburg et al., 2005).

In batch culture fermentation, the system is closed which facilitates the growth of microorganisms without adding or removing any material after inoculation. During this process the fermentation is carried out in sealed vessels inoculated with faecal suspensions maintained under anaerobic conditions. This system is convenient, easy and cheap to use and this allows a large amount of substrate or faecal sample to be tested at once in a short period of time. The system is useful for determining metabolic short chain fatty acid profiles produced by the gut microbiota through the metabolism of dietary components (Payne et al., 2012, Venema and van den Abbeele, 2013).

While the continuous culture fermentation models can be divided into single stage chemostats or multiple stage chemostats. The fermentation process requires a longer time to be achieved

and nutrients can be added or toxic substances can be removed while the fermentation occurs (Payne et al., 2012). Single stage chemostats represent the functions of the proximal colon while the multiple state chemostats have been further developed to represent the human colonic functions and metabolic actions that occurs in the proximal, transverse and distal colon. This enables the simulation of the horizontal colonic process in which the activities of the gut microbiota are studied at different conditions, representing the proximal colon (low pH and high carbohydrate) and distal colon (non-acidic, carbohydrate depleted)(Payne et al., 2012, Macfarlane et al., 1998).

### 1.6 Short chain fatty acid metabolism in the gut

Short chain fatty acids (SCFA) are the primary end products of saccharolytic fermentation of non-digestible carbohydrates (CHO) in the human gut where the main substrates of fermentation are the undigested starches that escape the stomach and the polysaccharides present in the plant cell wall (Macfarlane and Macfarlane, 2007b, Morrison and Preston, 2016). Although acetate, propionate and butyrate are the major SCFAs produced accounting for 85-95 % of the total SCFA produced in the colon, branched chain fatty acids such as iso-butyrate, iso-valerate and 2-methyl butyrate from valine, leucine and iso-leucine respectively are also produced form amino acid/protein fermentation (Cummings and Macfarlane, 1997, Cummings and Macfarlane, 1991). Due to the availability of carbon sources especially carbohydrate (CHO), the maximum concentration of SCFAs are produced in the proximal colon (Cummings and Macfarlane, 1997). When these carbon sources have been used up in the proximal colon there is a reduction in the availability of substrates as remaining food residues travel to the distal colon. This situation as well as colon transit time affects the amount of SCFA produced in the gut (Macfarlane and Macfarlane, 2007b). Furthermore, in addition to the colon transit time, host

related factors (such as diet consumed and nutrient availability) and other factors such as aging, stress, use of antibiotics, disease, secretions in the digestive tract, and also environmental factors such as the chemical composition in the gut, substrate availability, bacterial composition and competitive interactions among the different bacterial species in the gut are factors that influence the concentration of SCFA in the gut (Macfarlane and Macfarlane, 2007b, Cummings and Macfarlane, 1991, Morrison and Preston, 2016, den Besten et al., 2013).

### 1.7 Amino acid sources used for the study

Amino acids are organic compounds containing amine ($-NH_2$) and carboxyl (-COOH) functional group as well as a side chain R group which is specific to each amino acid. Amino acids are the building blocks of proteins i.e. they are substrates used for the synthesis of proteins and other nitrogenous compounds. Various studies have been able to show the roles of amino acids in maintaining a healthy gut (Wang et al., 2009, Reeds, 2000). Therefore, for the purpose of this research work we decided to look at the effect of various amino acid sources which provide nutrients for bacterial growth on the production of GABA in the gut and the sources used were peptone, tryptone, casaminoacids, yeast extract, L-cysteine and L-tryptophan.

#### 1.7.1 Peptone, Tryptone and Casamino acids

Peptone is a mix of soluble proteins formed in the early stage of protein breakdown during digestion. It is formed by partial hydrolysis or digestion of proteins by an acid or enzyme (proteolysis) therefore, the main role peptides found in peptone is to provide nutrients for bacterial growth (Amezaga and Booth, 1999) . Casamino acids are a mixture of amino acids and some peptides obtained from the acid hydrolysis of casein while tryptone is a mixture of peptides obtained from the digestion of casein by the protease trypsin. Casamino acids are similar to tryptone in that they are both formed by the digestion of casein but differ in that

casamino acids contain free amino acids and few peptides which are present due to the acid hydrolysis of casein but lacks tryptophan while tryptone has few oligopeptides that are formed from the digestion of casein by protease trypsin and are rich in tryptophan (Mueller and Johnson, 1941, Fraser and Powell, 1950).

### 1.7.2 L-tryphtophan and L-cysteine

L-tryptophan is an amino acid used in the biosynthesis of proteins and it contains a α-amine group, a α-carboxylic acid group and a side chain indole. It is also a precursor of the neurotransmitter serotonin and the hormone melatonin and it is essential for normal growth in infants and for nitrogen balance in adults (Slominski et al., 2002). L-trypthophan is similar to L-cysteine as they both can be obtained from diet which is high protein food sources. L-trypthophan cannot be synthesised by the body while L-cysteine can be biosynthesised by the body in required amount in the presence of methionine. L-cysteine is a non-essential sulphur containing amino acid and it plays important role in protein synthesis, detoxification and also metabolism of essential biochemical (Kredich, 2008).

### 1.7.3 Yeast extract

Yeast extract is a processed form of yeast and it is obtained by removing the cell wall and obtaining the cell content. It contains glutamic acid that can be used as amino acid source found in meat, cheese, vegetables etc. It is usually used as a food additive and serves as a source of nutrient for bacterial growth (Revillion et al., 2003).

## 2. Hypotheses

- The simple, cheap and easy colorimetric screening method on agar plates would allow us to identify easily bacteria with an active GAD system between thousands of colonies.
- Glutamate Decarboxylase system plays a major role in the GABA production and hence provides major health benefits for humans and animals.

## 3. Objectives

The objectives of this research work are:

- To develop a method that is cheaper, faster and more effective in easy identification of major GABA producers in the gut microbiota.
- To screen various lactic acid bacteria strains from the gut microbiota for the production of GABA.
- To determine GABA production in the gut as well as look into the behaviour and characteristics of the GABA producers in the gut.
- Identify conditions and signals that affect GABA production in the gut.

## 4. Thesis outline

**4.1 Chapter 1:** Literature review: Introduction to and overview of GABA production with particular attention to the gut microbiota, functions and metabolism.

**4.2 Chapter 2:** Colorimetric Screening test: An easy way of identifying Gamma aminobutyric acid (GABA) producers.

**4.3 Chapter 3:** Probiotic Potential of γ-aminobutyric acid (GABA) producing Lactic Acid Bacteria from gut microflora

**4.4 Chapter 4:** Modulation of the gut microflora γ-aminobutyric (GABA) and short chain fatty acids (SCFAs) production through feed in pH-controlled single stage continuous culture fermentation and static batch culture fermentation

**4.5 Chapter 5:** Effect of casaminoacids and L-cysteine on γ-aminobutyric acid (GABA) and short chain fatty acid (SCFA) production in the gut in a multiple stage continuous culture fermentation model

**4.7 Chapter 6:** General discussion, limitations and future perspectives

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# Chapter 2

# Colorimetric Screening test: An easy way of identifying γ-aminobutyric acid (GABA) producers.

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# Abstract

Due to the positive health effects of GABA, isolation of GABA producers has great biotechnological potential. The current study aims at the development of a rapid and easy method of screening bacteria with an active glutamate decarboxylase system (GAD) able to produce high levels of extracellular γ-aminobutyric acid (GABA). Currently, screening for GABA producers is performed with HPLC or GABAse assay which are relatively expensive and laborious methods.

To select strains with increased GAD activity a pre-screening method was established. Strains that were isolated from different environments were plated on a colorimetric screening plate containing 1.2g/l L-glutamic acid, 10 mg/l methyl red and 5 mg/l methylene blue; pH 6.0 and their colour changes was observed on the screening plates after 48 h. Colonies with bright green colour were then selected and assayed for GABA production.

Colorimetric screening is a cheaper and faster method for identifying GABA producers. There was a correlation between GABA produced from *Lactic acid bacteria* (LAB) isolated from different environment and colorimetric screening with an overall identification rate of 100% for GABA producers (>2.5 mM GABA concentration). This method can be applied to isolates from various microbial populations.

This study was the first to use a pH indicator-based technique as a pre-screening for GABA producers from various strains of LAB isolated from different environments and our finding is important for the optimization of GABA production.



**Keywords:** aminobutyric acid, glutamate decarboxylase system, colorimetric test.

# 1. Introduction

γ-aminobutyric acid (GABA) is a four carbon non-protein amino acid which functions as an inhibitory neurotransmitter in the mammalian brain (Hudec et al., 2014) while Glutamate decarboxylase (GAD) is pyridoxal-5-phosphate dependent enzyme that is catalysed by the decarboxylation of glutamate to GABA (Feehily and Karatzas, 2013). At low pH, the GAD system imports extracellular glutamate with the help of an antiporter in exchange for an extracellular GABA by consuming protons i.e. L-Glu + $H^+$ —> GABA + $CO_2$ (Karatzas et al., 2012). Several microorganisms have been found to possess the GAD system, and microorganisms with active GAD system are desirable as GABA produced by chemical synthesis is not suitable for mammalian use (Yang et al., 2006).

Studies have shown that orally administered GABA has tranquilizing and diuretic properties, as well as ability to relief depression, anxiety, sleeping disorders and reduce blood pressure (Siragusa et al., 2007, Distler et al., 2012, Tujioka et al., 2009, Padgett et al., 2012). GABA can be found in neural and non-neural tissues outside the brain including the gastrointestinal tract and it participates in regulating various neural signal pathways and physiological functions which has been widely studied and these studies have led to the development of GABA-enriched functional foods. GABA has been reported to regulate blood pressure in both hypertensive rats and human intervention trials (Hayakawa et al., 2004, Inoue et al., 2003, Tanaka et al., 2009). Hypertension is a major cardiovascular disease, therefore a reduction in the high blood pressure can help in controlling other related diseases and illnesses thus production of GABA and GABA enriched foods has an important health benefit for patients suffering from cardiovascular and other related illnesses. A variation i.e. increase or decrease in GABA production has been linked with various diseases such as Parkinson's, seizures, Alzheimer's as the GAD substrate L-glutamate stimulates and acts as an inhibitor in the neuron (Ting Wong et al., 2003, Battaglioli et al., 2003). GABA has also been found to help in the

prevention of sleeping disorders, mood disorders, depression and it improves immunity against stress and alcohol- related disorders as it protects against kidney diseases while activating the functions of the kidneys and liver (Ting Wong et al., 2003, Sasaki et al., 2006, Krystal et al., 2002, Bjork et al., 2001). It can also act against diabetes as it has been proven that GABA helps in insulin secretion (Adeghate and Ponery, 2002). GABA has been shown to slow down the proliferation of various cancer cells, and it is also considered to help in suppressing tumours (Schuller et al., 2008, Oh and Oh, 2004, Kleinrok et al., 1998). It has also been demonstrated that GABA can increase growth hormone production, it also improve the function of the thyroid hormone (Adeghate and Ponery, 2002, Wiens and Trudeau, 2006, Xie et al., 2014).

Some reasons of GABA being effective in terms of its major health benefits could possibly be due to its relaxing effect on users by inhibiting the nerves cells in the brain from receiving much stimulatory effect which in turns causes the patients to maintain a relatively calm and relaxed state (Harris and Allan, 1985). The GABA receptors ($GABA_A$ and $GABA_B$) have been implicated in the various physiological activities of GABA. GABA functions by binding to its specific receptors and it activates them to regulate the chloride ion distribution between the nerve cell membranes i.e. they increase membrane conductance of chloride ion in the nerve cells. The activated GABA receptors ($GABA_A$ and $GABA_B$) cause an influx of chloride ion into the nerve cells by opening the chlorine ion channels and thus causing hyperpolarisation or reducing neural excitability in the cell membrane thereby reducing the activity of the cell and thus lowering blood pressure (Ma et al., 2015, Vaz et al., 2015). Also, studies have shown that GABA receptors are expressed and activated in tumour cells and also GABA might be involved in the migration of tumour cells in a way that is similar to that of chemokines as GABA through the GABA receptors affects every stage of cell development such as proliferation, migration and differentiation therefore levels of GABA receptors are upregulated in cancer cells thus

raising the possibility of reducing the activities of the cells by manipulating the GABA receptors. (Joseph et al., 2002, Ortega, 2003, Takehara et al., 2007, Watanabe et al., 2006).

Therefore, due to these physiological effects, the need to produce GABA enriched functional foods has been on the increase and this has led scientists to study the bacterial glutamate decarboxylase system (GAD), that is mainly responsible for the production of GABA in fermented foods. Different studies on GABA have shown various techniques such as chromatography based methods used in quantifying the amount of GABA produced. These methods are time consuming and quite expensive to run per sample therefore, this study aimed in developing a pH indicator-based technique that is easier and cheaper to screen and select as many colonies of bacteria at once which have an active GAD system. Further to the development of the method we also tested its usage in microbiota from various environments and identified the rate of GABA producers in each of these environments.

The colorimetric screening method was modified from Shi et al. (2014) which it was used to improving GAD activity of mutant strains of *Lactobacillus brevis* by expanding the pH range of activating the *gadB1* to near neutral pH and then improving the synthesis of GABA with the mutant *gadB1* in *Corynebacterium glutamicum*. The method is a colorimetric screening, with a pH indicator used to determine microbes that are able to increase the pH of the colony. It is a cheaper and rapid method for the isolation of strains that produce high levels of GABA. It can be used to screen many colonies at a time which is less time consuming. In principle, because the GAD reaction consumes protons and hence increases the pH, therefore, a sensitive pH indicator could be used to detect the pH change. Methyl red methylene blue was therefore used for this study as it gives grey colour at a pH range of 4.2 to 6.2 and green colour at a pH range of 6.2 to 7. In theory, other amino acid decarboxylase systems could also be detected with this test giving many false positives. However, the GAD system is the most potent amino acid decarboxylase system which could coincide with the early appearance of green colonies

that could be identified. Therefore, since the GAD system is known to be extremely active it might be possible to distinguish from the other amino acid decarboxylase systems that could also increase the pH of the colony but at a much slower rate.

## 2. Materials and Methods

To select the strains with increased GAD activity, a two-step pre-screening procedure was established. Firstly, strains isolated from different environments were plated on colorimetric screening plates containing 1.2g/l L-glutamic acid, 10 mg/l methyl red and 5 mg/l methylene blue; pH 6.0 and the colour change of the colonies was observed after 48 h. Colonies showing a bright green colour were then selected and assayed for GABA production. All compounds used were purchased from Sigma Aldrich (Dorset,UK) except otherwise stated.

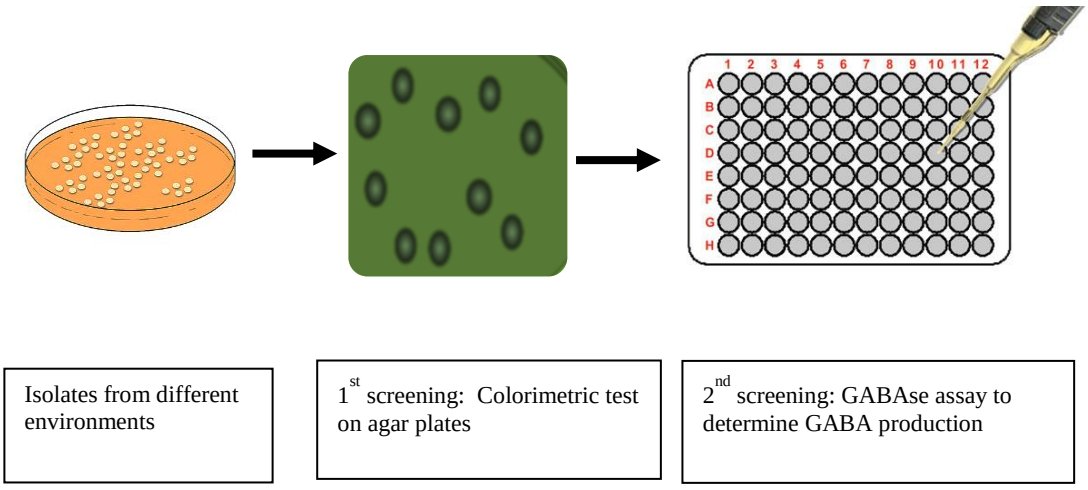



**Fig 1:** Schematic diagram of the colorimetric screening test showing the two-step pre-screening procedure to determine GABA producers with higher GAD activity

### 2.1.1 Isolation and identification of LAB strains isolated from the gut

Ten percent (w/v) of faecal slurry was prepared by taking fresh faecal samples from a healthy volunteer (not received antibiotics for 6 months) mixed with the stomacher for 2 min in phosphate saline buffer (pH 7.4). Decimal dilutions of the samples were prepared and 100 µl of each dilution was spread on DE Man-Rogosa and Sharp (MRS) agar and incubated anaerobically at 37$^{o}$C for 48 h. Colonies were selected from the appropriate dilution and purified on MRS agar and also incubated anaerobically at 37 $^{o}$C for 48 h. Pure cultures were stored at -80 $^{o}$C in cryovial tubes containing MRS broth with the addition of 7% Dimethyl sulphoxide (DMSO). Each colony was sub-cultured, selected and identified based on their catalase activity in hydrogen peroxide. Isolates were also checked if they are Gram-positive by using gram staining technique and also biochemically using the API 50 CH identification kit along with API 50 CHL medium (Biomerieux,UK) for the identification of LAB.

### 2.1.2 Isolation and identification of LAB strains isolated from the cheese and olives (Pavli et al., 2016, Argyri et al., 2013).

Olive samples from Spanish style green olives of cv. Conservolea and Halkidiki as well as black olives from cv. Conservolea and Kalamata were collected from six processing plants in different regions of Greece. The olive samples were directly taken from the fermentation vessels at the end of the fermentation process to the laboratory for further analysis. Also, traditional Greek products such as feta cheese, manuori cheese and xerotyri cheese were obtained from local markets in Greece.

Samples of 25 g were weighted aseptically, added to 225 ml quarter strength Ringer's solution (LABM, Lancashire, UK) and homogenized in a stomacher (Stomacher 400 circulator, SEWARD LIMITED, Norfolk, UK) for 60 sec at room temperature. Decimal dilutions were

prepared and 1 ml of the sample was mixed on De Man-Rogosa and Sharpe agar (OXOID, Hampshire, UK). MRS Agar was used for selection and quantification of LAB population and was incubated at 30°C for 48- 72 h. 20% of the colonies were randomly selected and purified from each sample from the appropriate dilution of the growth medium. Pure cultures were stored at -80°C in MRS broth supplemented with 20% (v/v) glycerol (APPLICHEM, Darmstadt, Germany).

### 2.2 Bacterial strains

Strains were isolated from different environments namely, fermented olives, cheese and faeces. The isolates were streaked onto agar plates and grown anaerobically. Three colonies of each isolate were taken from the agar, plated and inoculated in 3 ml of MRS broth and then incubated anaerobically for 48 h which is the time required for the strains to reach the stationary phase of growth. All experiments were carried out in triplicate and compounds used were all purchased from Sigma Aldrich, (Dorset,UK) except otherwise stated.

#### 2.2.1 Colorimetric screening for GABA production

The agar plates were prepared using MRS and also compared with Wilkins Chalgren anaerobe agar (Oxoid) and Nutrient agar (Oxoid). The agar plates were prepared containing 1.2g/l L-glutamic acid, 10 mg/l methyl red and 5 mg/l methylene blue. The pH of the media was adjusted to 6.0 and then 10µl of the overnight broth culture was plated and incubated anaerobically at 37 $^{o}$C for 48 h. The changes in the colour of the colonies was observed over the time period.

#### 2.2.2 GABA quantification

GABA quantification was performed according to Tsukatani et al. (2005) and Karatzas et al. (2010). This was done by first growing the strains in MRS broth (3 colonies in 3 ml of MRS broth) overnight under anaerobic conditions and then inoculating the culture from the first overnight for a second culturing for 48 h in MRS broth (50 µl of culture into 5 ml MRS broth

containing 10 mM L-glutamate). The second culture was subsequently centrifuged at 11337×g for 10 min and the supernatants were stored in Eppendorf tubes and kept frozen until they were needed for the assay. GABAse master mix was prepared containing 80 mM Tris (base) amino methane, 2 mM α-ketoglutarate,750 mM sodium sulphate ($Na_2SO_4$), 10 mM dithiothreitol (DTT), 1.4 mM beta-nicotinamide adenine dinucleotide phosphate (NADP) and 0.3 g/l GABASE enzyme from *Pseudomonas fluorescens* (Sigma Aldrich, Dorset, UK) in sterile water was prepared. Ninety µl of the master mix was placed in the wells of a 96-well plate and subsequently, 10 µl of the defrosted supernatant was added. GABA standard solutions containing a known amount of GABA (0, 2, 4, 6, 8 and 10 mM) were prepared and run alongside the samples. The plate was run at wavelength of 340 nm at 37$^{o}$C for 3 h using the Sunrise plate reader and data was collected using Magellan software (TECAN, Mannedorf, Switzerland). The readings were taken at intervals of 2 min and the results were analysed using Microsoft excel.

# 3. Results

### 3.1 GABA producing isolates from various environments

The GABA producers were *Lactobacillus plantarum* (33 strains), *Lactobacillus fermentum* (11 strains), *Lactobacillus brevis (1 strain), Lactobacillus salivarus* (1 strain), *Leuconostoc lactis* (2 strains) and the non-producers are majorly *Lactobacillus paracasei* (22 strains), *Lactobacillus acidophilus* (6 strains*).* The growth media used for isolation and culturing was selective for the strains that we were able to isolate and also influenced the GABA concentration from the isolated strain. The strains producing GABA that were isolated on MRS agar were majorly *Lactobacillus plantarum* and *Lactobacillus paracasei* similarly to the non-producers, while the isolates obtained on Wilkins charlgren and Nutrient agar were majorly *Lactobacillus fermentum* as GABA producers and *Lactobacillus acidophilus* as the non-producers We found that 87 % of the GABA producers also showed a positive response to the colorimetric test..

| Codes | Strains | Major GABA production (>2.5mM) | Colorimetric test | Environment isolated from |
|---|---|---|---|---|
| 2A | *Lactobacillus pentosus* | + | + | olives |
| 3A | *Lactobacillus paracasei* | + | + | olives |
| 6A | *Lactobacillus pentosus* | + | + | olives |
| 8A | *Lactobacillus plantarum* | + | + | olives |
| 10A | *Lactobacillus plantarum* | + | + | olives |
| T47 | *Lactobacillus paraplantarum* | + | + | cheese |
| T71 | *Lactobacillus plantarum* | + | + | cheese |
| T73 | *Lactobacillus plantarum* | + | + | cheese |
| M92 | *Lactobacillus plantarum* | + | + | gut |
| M93 | *Lactobacillus plantarum* | + | + | gut |
| M88 | *Lactobacillus plantarum* | + | + | gut |
| M89 | *Lactobacillus plantarum* | + | + | gut |
| M87 | *Lactobacillus plantarum* | + | + | gut |
| M86 | *Lactobacillus plantarum* | + | + | gut |
| M84 | *Lactobacillus plantarum* | + | + | gut |
| M85 | *Lactobacillus plantarum* | + | + | gut |
| M77 | *Lactobacillus plantarum* | + | + | gut |
| M75 | *Lactobacillus plantarum* | + | + | gut |
| M73 | *Lactobacillus plantarum* | + | + | gut |
| M95 | *Lactobacillus plantarum* | + | + | gut |
| M22 | *Lactobacillus plantarum* | + | + | gut |
| M94 | *Lactobacillus plantarum* | + | + | gut |
| M79 | *Lactobacillus plantarum* | + | + | gut |
| W56 | *Lactobacillus plantarum* | + | + | gut |

| | | | | |
|---|---|---|---|---|
| W52 | *Lactobacillus plantarum* | + | + | gut |
| W47 | *Lactobacillus plantarum* | + | + | gut |
| W33 | *Lactobacillus fermentum* | + | + | gut |
| W17 | *Lactobacillus fermentum* | + | + | gut |
| W54 | *Lactobacillus plantarum* | + | + | gut |
| W76 | *Lactobacillus plantarum* | + | + | gut |
| W74 | *Lactobacillus fermentum* | + | + | gut |
| W70 | *Leuconostoc lactis* | + | + | gut |
| N40 | *Lactobacillus fermentum* | + | + | gut |
| N28 | *Lactobacillus salivarus* | + | - | gut |
| N98 | *Lactobacillus fermentum* | + | + | gut |
| N95 | *Lactobacillus fermentum* | + | + | gut |
| N83 | *Lactobacillus fermentum* | + | + | gut |
| N13 | *Lactobacillus.brevis* | + | + | gut |
| N20 | *Lactobacillus fermentum* | + | + | Gut |

| **Codes** | **Strains** | **GABA production (>detection limit)** | **Colorimetric test** | **Environment isolated from** |
|---|---|---|---|---|
| 1A | *Lactobacillus paracasei* | - | - | olives |
| 4A | *Lactobacillus plantarum* | + | + | olives |
| 5A | *Lactobacillus pentosus* | - | + | olives |
| 7A | *Lactobacillus pentosus* | - | + | olives |
| 9A | *Lactobacillus rhamnosus* | - | + | Reference strain |
| 11A | *Lactobacillus shirota* | + | - | Reference strain |
| T571 | *Lactobacillus plantarum* | - | - | cheese |
| L35 | *Lactobacillus sakei* | + | + | cheese |
| L41 | *pending* | - | + | cheese |

| | | | | |
|---|---|---|---|---|
| L49 | *Lactobacillus pentosus* | - | + | cheese |
| L119 | *Lactobacillus plantarum* | + | + | cheese |
| L125 | *Lactobacillus plantarum* | + | + | cheese |
| L132 | *Lactobacillus plantarum* | + | + | cheese |
| T48 | *Lactobacillus plantarum* | + | - | cheese |
| L363 | *pending* | - | - | cheese |
| L207 | *Lactobacillus paraplantarum* | + | - | cheese |
| M64 | *Lactobacillus paracasei* | - | - | gut |
| M63 | *Lactobacillus paracasei* | - | - | gut |
| M57 | *Lactobacillus paracasei* | - | - | gut |
| M38 | *Lactobacillus paracasei* | - | - | gut |
| M35 | *Lactobacillus paracasei* | - | - | gut |
| M98 | *Lactobacillus plantarum* | - | - | gut |
| M70 | *Lactobacillus paracasei* | - | - | gut |
| M69 | *Lactobacillus paracasei* | - | - | gut |
| M66 | *Lactobacillus paracasei* | - | - | gut |
| M65 | *Lactobacillus paracasei* | - | - | gut |
| M59 | *Lactobacillus paracasei* | - | - | gut |
| M11 | *Lactobacillus paracasei* | - | - | gut |
| M96 | *Lactobacillus plantarum* | + | + | gut |
| M56 | *Lactobacillus paracasei* | - | - | gut |
| M47 | *Lactobacillus paracasei* | - | - | gut |
| M44 | *Lactobacillus rhamnosus* | - | - | gut |
| M41 | *Lactobacillus paracasei* | - | - | gut |
| M40 | *Lactobacillus paracasei* | - | - | gut |
| M39 | *Lactobacillus paracasei* | - | - | gut |
| M80 | *Lactobacillus plantarum* | - | - | gut |

| | | | | |
|---|---|---|---|---|
| M36 | *Lactobacillus paracasei* | - | - | gut |
| M19 | *Lactobacillus paracasei* | - | - | gut |
| M15 | *Lactobacillus paracasei* | - | - | gut |
| W84 | *Lactobacillus plantarum* | - | + | gut |
| W40 | *Lactobacillus acidophilus* | - | + | gut |
| W44 | *Lactobacillus fermentum* | + | - | gut |
| W35 | *Lactobacillus fermentum* | + | - | gut |
| W28 | *Lactobacillus plantarum* | - | + | gut |
| W19 | *Lactobacillus plantarum* | + | + | gut |
| W58 | *Lactobacillus plantarum* | - | - | gut |
| W93 | *Lactobacillus acidophilus* | - | - | gut |
| W88 | *Lactobacillus fermentum* | + | + | gut |
| W59 | *Lactobacillus fermentum* | - | - | gut |
| W3 | *Lactobacillus paracasei* | - | - | gut |
| W5 | *Lactobacillus acidophilus* | - | - | gut |
| W86 | *Lactobacillus acidophilus* | - | - | gut |
| W68 | *Lactobacillus acidophilus* | - | - | gut |
| W66 | *Lactobacillus acidophilus* | - | - | gut |
| W63 | *Lactobacillus acidophilus* | - | - | gut |
| N52 | *Lactobacillus paracasei* | - | - | gut |
| N65 | *Leuconostoc lactis* | + | + | gut |

**Table 1:** Shows the identification of LAB strains using the API 50CH identification kit (Biomerieux), the environment in which they were isolated from, GABA production and also colorimetric screening result.

### 3.2 Colorimetric screening test

From the colorimetric test, 86 % of the strains that showed vivid green colour (pH 6.2-7) showed high GABA concentration assayed through GABAse (i.e. 46 out of the 55 GABA producers showed positive correlation to the colorimetric test). The colorimetric screening test showed a 100 % (30 out of the 30 strains of major GABA producers) correlation with strains that showed vivid green colouration in the colorimetric screening test and major GABA producers (with >2.5mM GABA concentration ) identified during the GABAse assay (Fig 3). The strains that showed a very active GAD system gave a very dark green colouration especially in the centre of the colonies and 46 of the strains were also high GABA producers with the exception of a few (6) strains for example in Fig. 2, *L. rhamnosus* 9A seemed to show a dark green coloration but are non-GABA producing (Fig 3). Furthermore, there was 100% identification rate for the GABA producers (> 0.7 mM) and the major GABA producers (>2.5mM) for the strains isolated on MRS, Nutrient and Wilkins charlgren agar.

Also, there was no GABA production from selected GABA producing and non-GABA producing strains that were cultured in nutrient broth (fig 5) and Wilkins charlgren broth (fig 6)

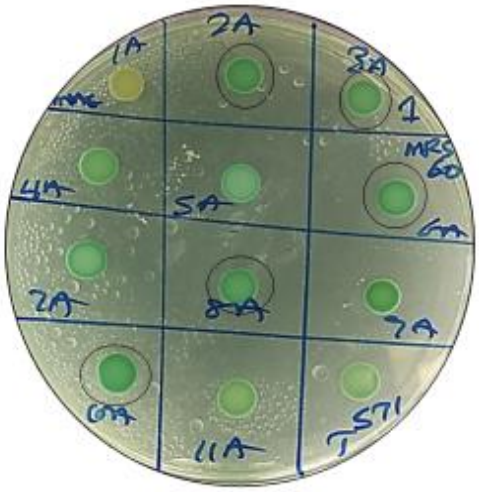
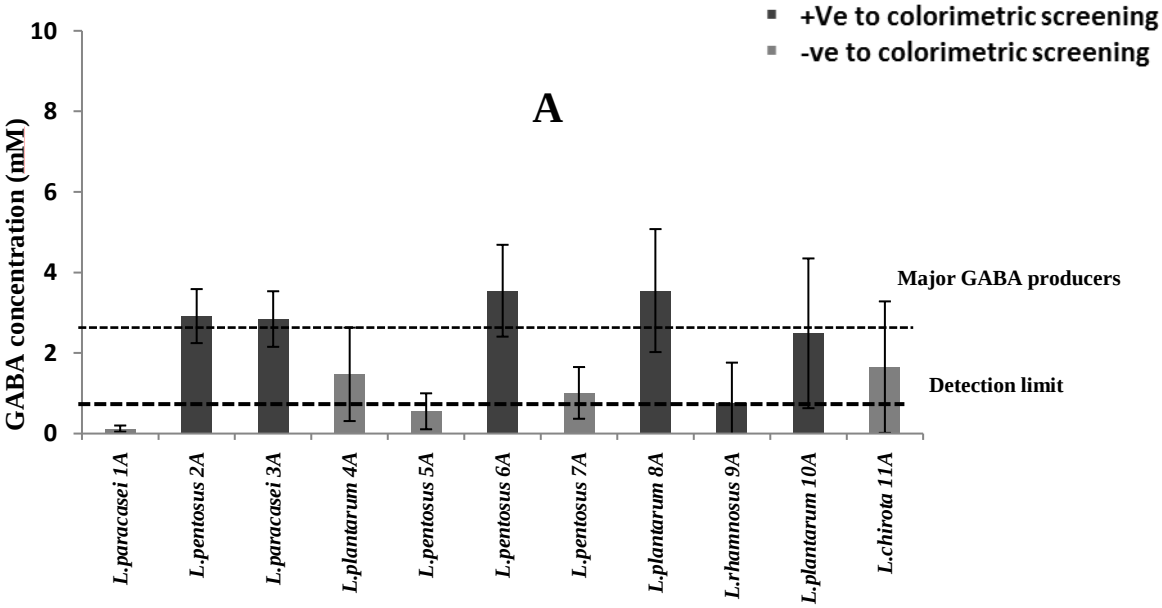



**Fig 2: C**olorimetric screening isolates from various environments. The colour changes for the pH indicator methyl red methylene blue (MM) were purple at a pH range of 1-4.2, grey at a pH range of 4.2-6.2 and green at a pH range of 6.2 and higher. The colonies with black circles show the GABA producers which were also positive to the colorimetric test.



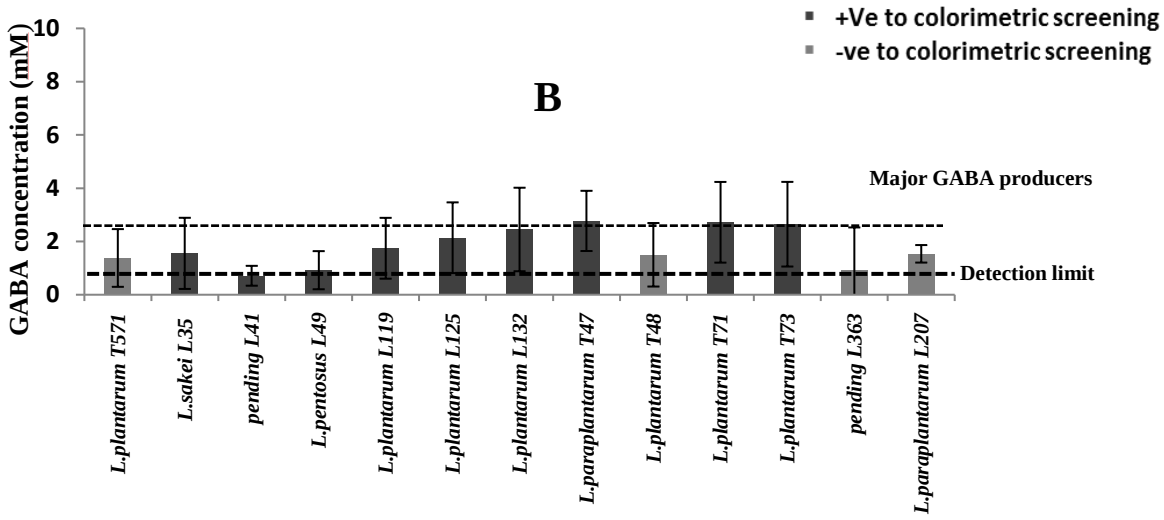
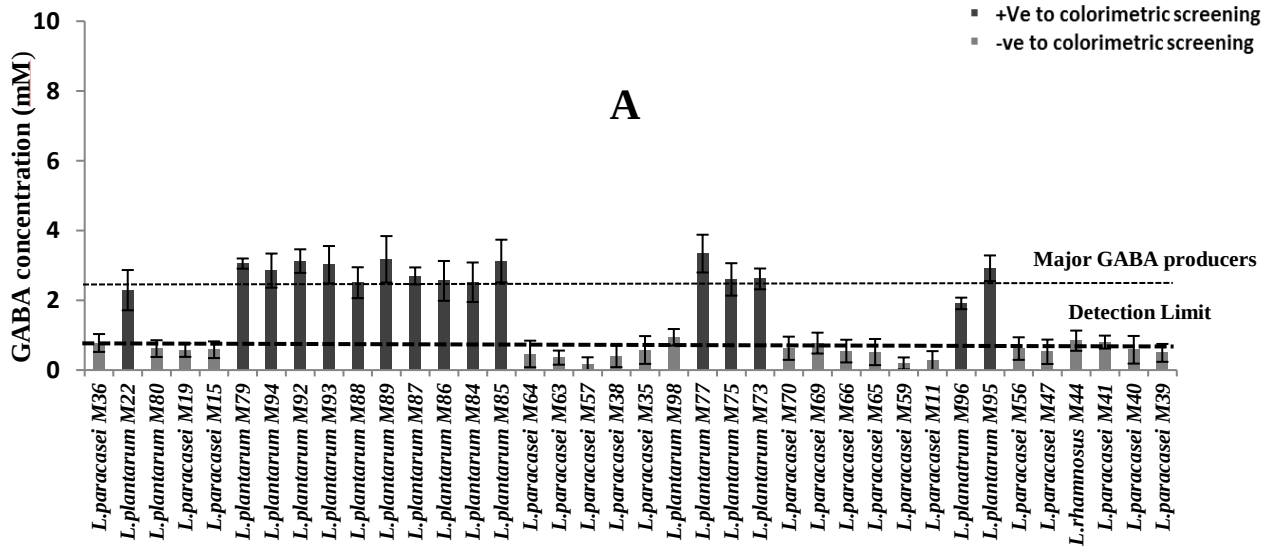



**Fig 3: C**olorimetric screening and GABA production (A) Strains isolated from fermented olives (B) strains isolated from cheese (Error bars represent standard deviations from 3 independent replicates). The strains that showed vivid green colour are represented with dark bars while strains that did not show green colour are represented with lighter bars. All the strains that were strong GABA producers (GABA levels >2.5mM) also produced green colonies. Fifteen out of the 24 strains produced bright green colouration resulting in an identification rate of >83% for the GABA producers (>0.7mM) and a 100% identification rate for the major GABA producers (>2.5mM).



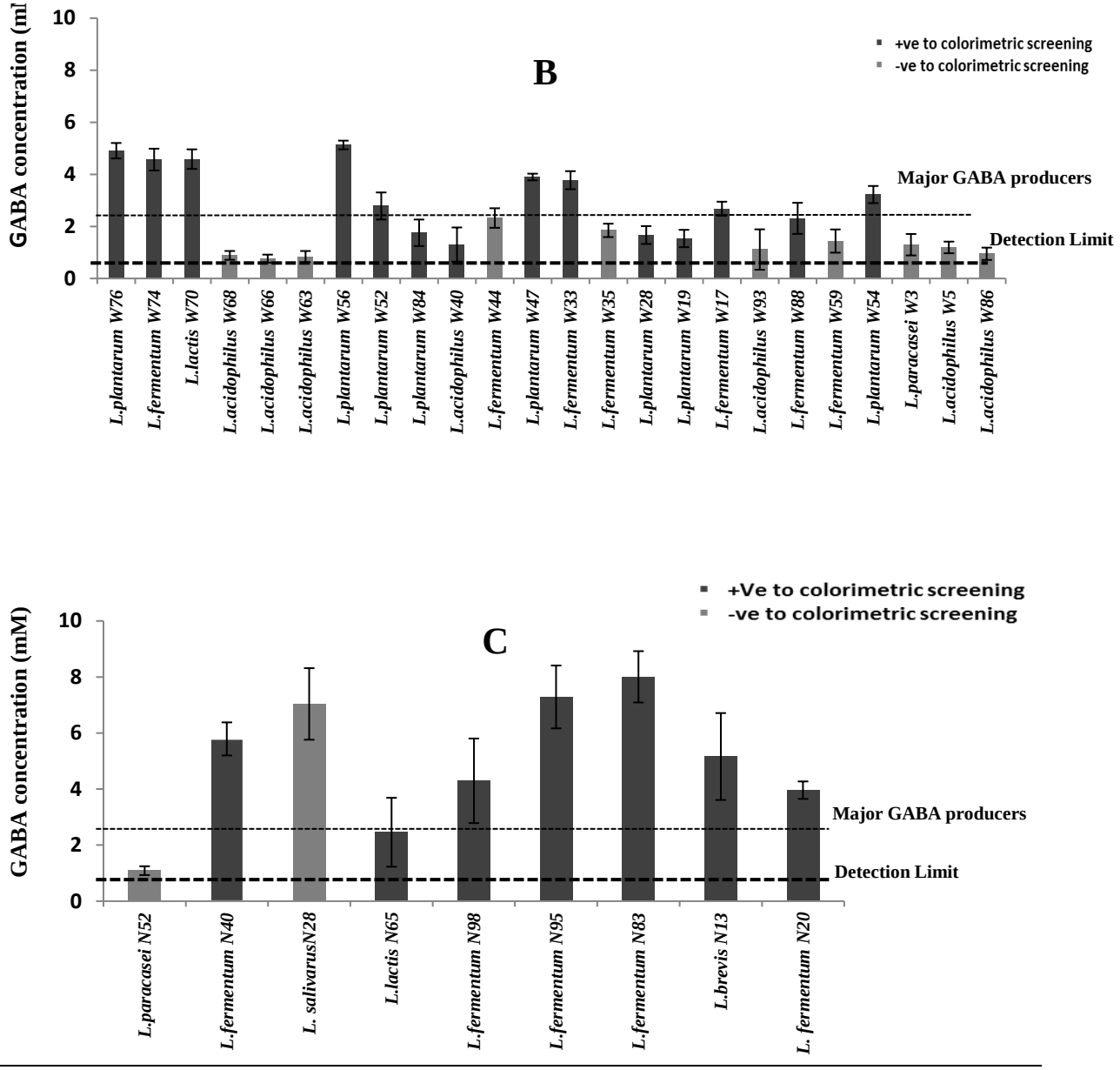



**Fig 4: C**olorimetric screening and GABA production for strains isolated from faecal samples. Strains used were isolated on MRS agar (A), Wilkins Chalgren agar (B) or Nutrient agar (C) while error bars represent standard deviations from 3 independent replicates. The strains that showed vivid green colour are represented with dark bars while strains that did not show green colour are represented with lighter bars. Strong GABA producers were those that produced >2.5mM GABA while GABA producers those with > 0.7 mM GABA.

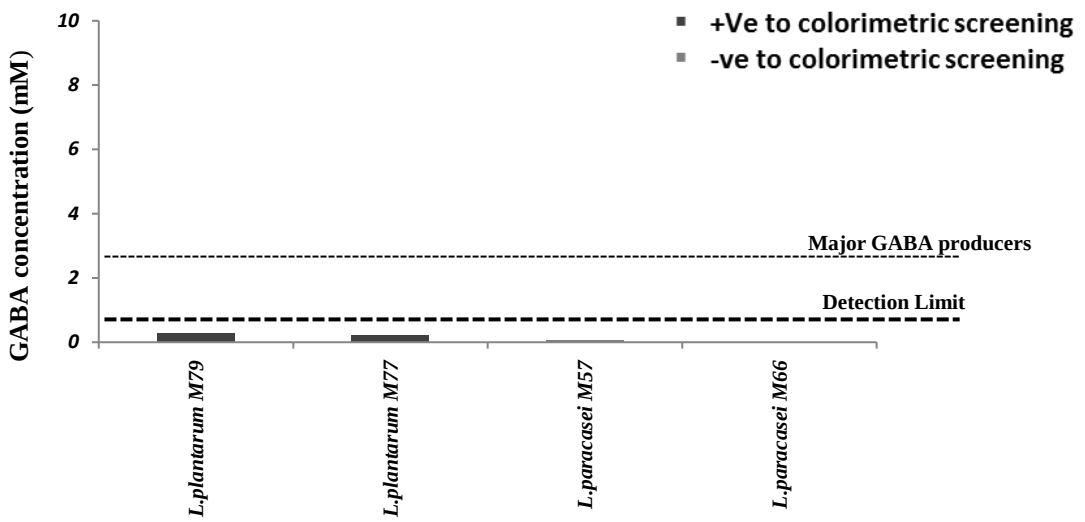



**Fig 5: C**oncentration of GABA produced using Nutrient broth. Two strains each out of the GABA producing and Non-GABA producing isolates were randomly selected from faecal samples. It shows there was no GABA production (> 0.7 mM) in the strains selected.

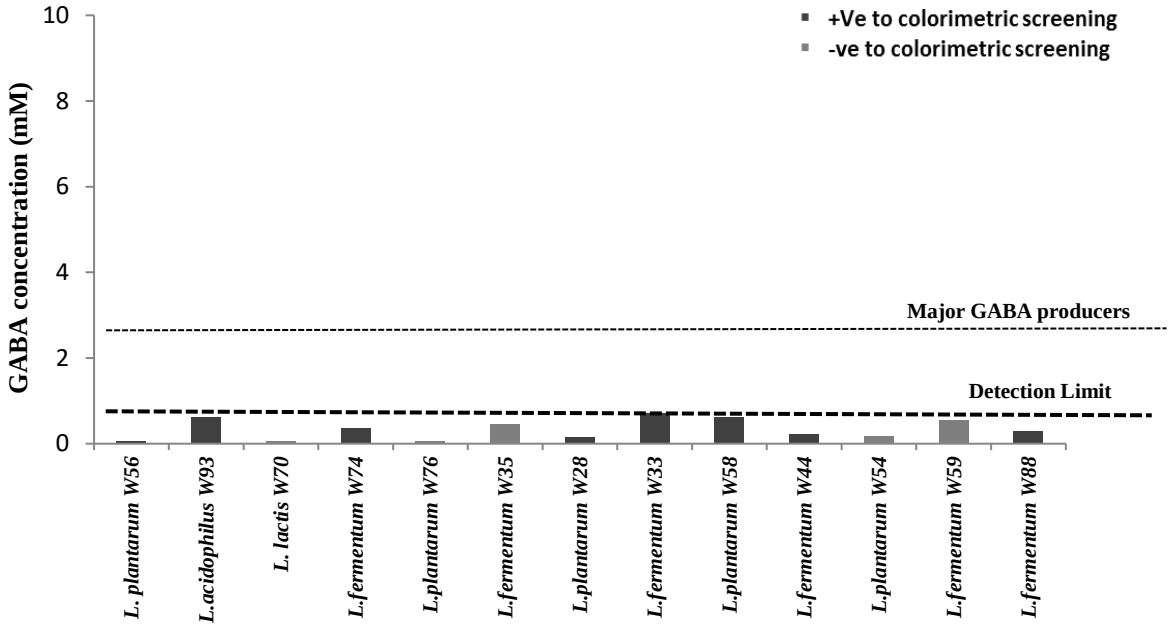



**Fig 6: C**oncentration of GABA produced using Wilkins charlgren broth. The samples were taken randomly from faecal samples it shows there was no GABA production (> 0.7 mM) in the strains selected .There was no GABA production using wilkins charlgren broth.

### 3.3 Correlation between acid resistance and GABA production

The glutamate decarboxylase system is a mechanism of acid resistance used by microorganisms. From our result, (Fig 7) *Lactobacillus plantarum* and *Lactobacillus shirota* (reference strain) shows more sensitivity to acid with 4 log reduction of CFU/ml. Although there was GABA production with the strains there was no correlation between GABA production and acid resistance (Fig 8).

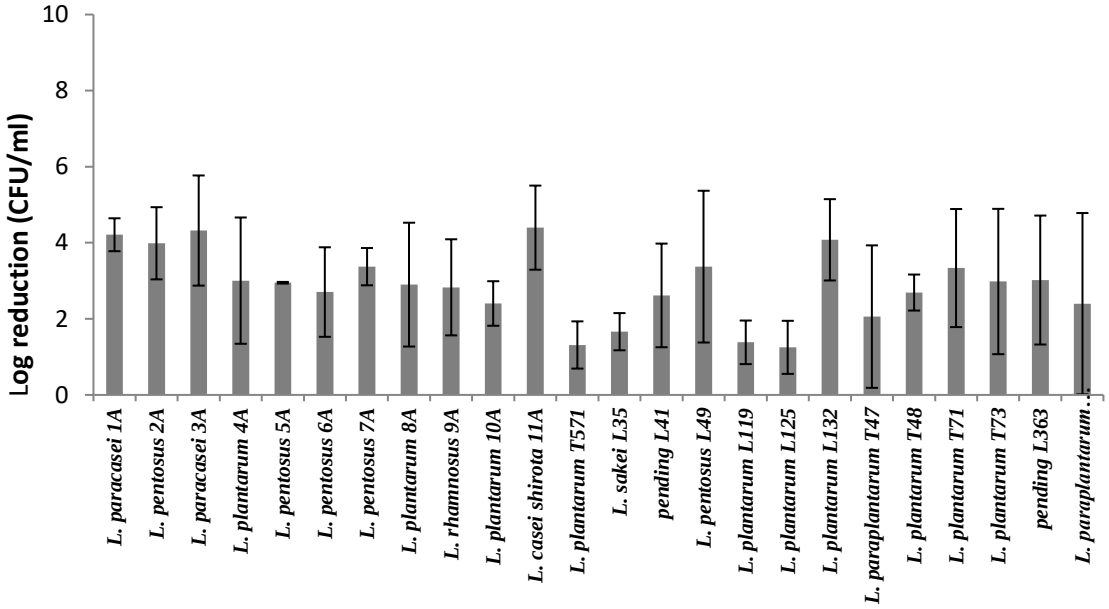



**Fig 7:** Log reduction in cfu/ml of olives and cheese strains when challenged with 1M HCl for 1 h at pH 2.5

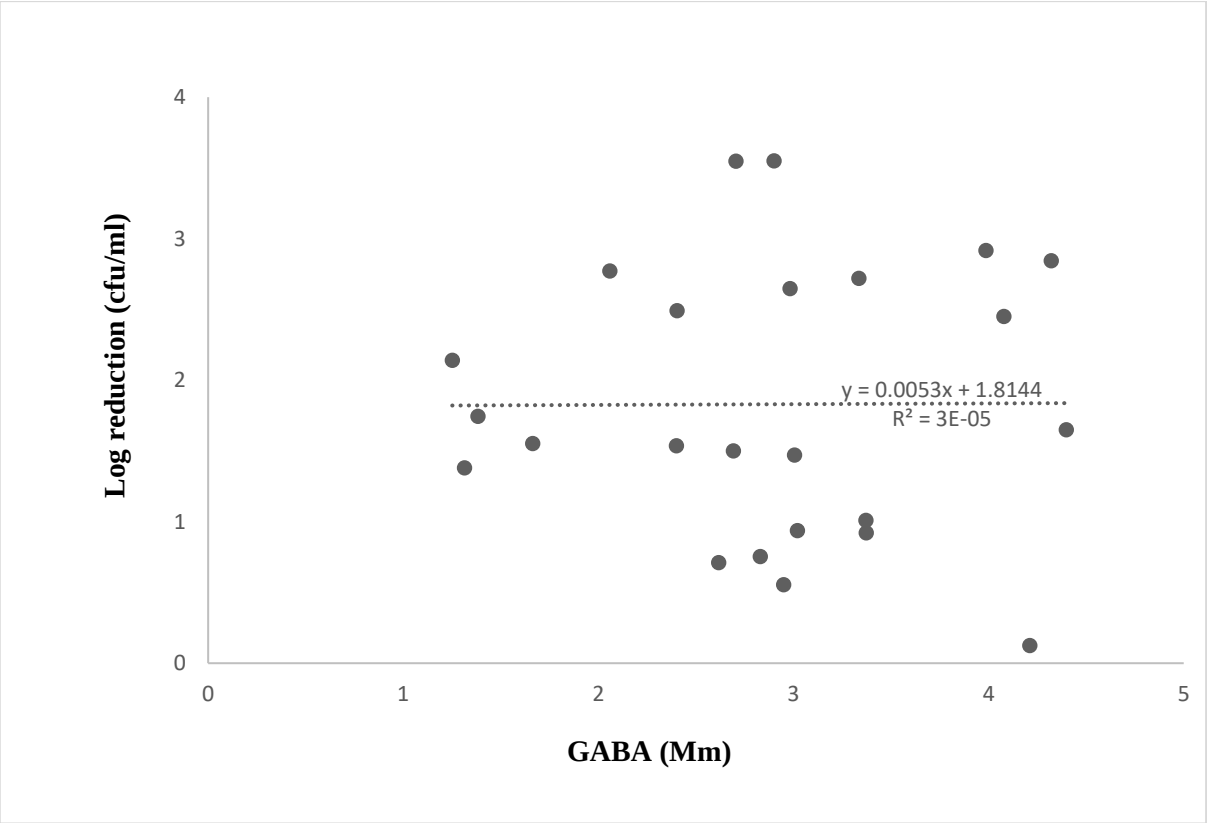



**Fig 8:** Correlation between GABA production and acid resistance which shows no linear correlation.

### 3.4 Correlation between colorimetric screening and GABA production.

These experiments were performed to identify if a colorimetric test could be used as a pre-screening to limit the number of strains that need to be tested by GABAse assay. We used strains isolated from two different environments (Fig 3; olives and cheese), and we decided to investigate also members of the gut microbiota isolated from faecal samples (Fig. 4). Therefore, we found that 67 % of the olive isolates, 70 % of cheese isolates and 65 % of faecal isolates which are GABA producers also showed a bright green coloration with the colorimetric screening test.

The strains were isolated on different culture media (MRS agar, nutrient agar and Wilkins Charlgren agar) and samples for GABAse were grown on MRS culture broth. To further

validate the test, we selected some GABA producing and non-producing strains from the colorimetric test and cultured them in Nutrient and Wilkins charlgren broth for GABA production. We found that the strains cultured in Nutrient broth (fig 5) and Wilkins charlgren broth (fig 6) did not produce any GABA which shows that nutrient broth and wilkins charlgren broth do not support the production of GABA from these isolates.

Isolates from all the different environments with colonies that gave very bright green coloration also produced GABA (> 0.7 mM) and this test gave an overall identification rate of above 100 %. Although there were no false positive (give green coloration, non-GABA producers) and 37 % false negatives (no green coloration, GABA producer) for the test on isolates from olives and 31 % false negatives from cheese and 35 % false negatives on isolates from the gut. However, isolates which give false +ves and –ves results are few compared to isolates which are positive to the test.

# 4. Discussion

GABA is a major inhibitory neurotransmitter of the central nervous system and existing research on its beneficial effects has increased the interest of researchers into optimizing its production. Therefore, to identify GABA producing isolates from a large microbial population an accurate and rapid method is important for differentiating the levels of GABA produced and also screening of GABA producing microorganisms. At present, chromatography-based techniques such as amino-acid analysis, thin-layer chromatography, liquid chromatography, gas chromatography or enzymatic-based techniques are used in quantifying the levels of GABA. These methods are tedious and time-consuming based on the time spent on sample preparation and the techniques also require a high level of workload which is not fast or economical for

screening GABA producing microorganisms. Hence, we decided to develop a pre-screening rapid and economical method for identifying GABA producers before the use of these chromatography-based techniques.

GABA is produced by the decarboxylation of glutamate using the GAD protein with the help of an antiporter. The GAD system of Lactic acid bacteria shows optimum activity at a pH of 4.0-5.0 (Shi et al., 2014, Yang et al., 2006). In this study, the pH indicator was used to observe the pH changes in the colorimetric agar plates as intracellular pH changes when the GAD is active. Therefore, with these colour changes we can easily identify isolates with a high GAD activity.

The feasibility of this method was tested using isolates from different environments cultured in different growth media and then further used the GABAse assay (GABA quantification method) to verify the correlation between colorimetric screening and GABA production. We found that 52 out of the 78 GABA-producing isolates from the different environments showed a positive result with the colorimetric test (66 %). This means that there were 26 false negatives (33 %) out 78 GABA producers that could not be detected. Furthermore, from the 52 isolates that were positive to the colorimetric test, there was no false positives i.e. isolates which showed a bright green coloration on the colorimetric plates but were not GABA producers. This suggests that the test can be used on a wider range of bacteria isolated from different sources or environments.

According to Dhakal et al. (2012), media composition is one of the factors affecting GABA production therefore we decided to test how efficient the method is by isolating the strains on different growth media (MRS agar, Nutrient agar and Wilkins Charlgren agar) containing the pH indicator. Therefore, from the results (fig 3A, 3B, & 4A), 16 of the 38 isolates on MRS agar (42 %), 7 of the 9 isolates on nutrient agar (78 %) as well as 14 of the 24 Wilkins Charlgren

agar (58 %) showed a bright green colouration and also are GABA producers. Also, MRS is a rich growth media which is selective for isolation of *Lactobacillus* therefore we were able to get 38 isolates using MRS compared to the other two growth media used. In addition, the isolates from MRS, Nutrient and Wilkins Charlgren agar when cultured in MRS broth (Fig. 4A, B & C) for GABA production yielded a high concentration of GABA but when cultured in Wilkins Charlgren (Fig. 5) and nutrient broth (fig 6) respectively there was no GABA production and this could possibly be as the result of the high levels of glucose/sugars in the MRS broth as glucose is essential as a carbon source for the metabolic process of fermentation of microorganism (Park et al., 2013). When fermentation occurs the internal pH of the cell is lowered thereby reducing acidification in the cytoplasm which in turn increases the GAD activity and hence increasing the amount of GABA produced in the cell which was observed with the GABA producing strains (Cotter et al., 2001). The GAD is used by microorganisms as a mechanism of acid resistance, but the result (fig 8) showed no correlation between the acid resistance of the isolates and GABA concentration of each strain and this could be explained that different strains respond differently under acidic conditions while the reference strain *L. casei shirota* (11A) which is generally used a probiotic shows the most sensitivity to acid resistance.

Finally, the pre-screening method using the colorimetric test is cost effective, it is also less laborious and saves a lot of time when compare to other chromatographic based techniques of GABA quantification. The test is cheap, fast and easy to carry out as a preliminary test to screen out GABA producers from non- GABA producers before other quantitative techniques for GABA production such as GABAse assay or chromatography-based methods are done as the quantitative techniques for GABA production could be quite expensive when having to measure GABA in so many samples.

# 5. Conclusion

GABA is used as a desirable bioactive component used in the food, feed and pharmaceutical companies because of their beneficial physiological function therefore, the colorimetric screening test developed is a cheaper, rapid and simpler method for identifying and pre-screening GABA producers form a population of isolates from various environments. Eighty-six percent of the LAB strains isolated from different environments were positive to both GABAse assay and colorimetric screening test with 100 % overall identification rate on the colorimetric test for major GABA producers (>2.5 mM). Therefore, this method can be applied to isolates from various microbial populations and it is a very useful method that can be used to meet the high demand in sampling frequency of GABA producing strains.

# 6. Acknowledgement

The authors would like to thank all colleagues at the Microbiology research team, Food and Nutritional Sciences, University of Reading, Society for Applied Microbiology (SFAM) for the studentship grant given. The Hellenic Agricultural organization DEMETER, Greece and Microbiology and Biotechnology laboratory group, Agricultural University of Athens for providing the strains used in this study.

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# Chapter 3

# Probiotic Potential of γ-aminobutyric acid (GABA) producing Lactic Acid Bacteria from gut microflora

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# Abstract

Thirty-eight (38) strains of Lactic acid bacteria isolated from the human gut were evaluated for their probiotic potential with *Lactobacillus plantarum* and *Lacctobacillus paracasei* being the most dominant of the isolated strains (18 *Lactobacillus plantarum*, 19 *Lactobacillus paracasei* and 1 *Lactobacillus rhamnosus*) while *Lactobacillus rhamnosus GG* and *Lactobacillus casei Shirota* were also studied as reference strains. Series of in-vitro tests such as survival (in acid and bile), acidification, safety (antimicrobial, biofilm formation and antibiotic activity) and GABA production were carried out for the selection of appropriate probiotic candidates. *L. plantarum* isolates produced a high GABA concentration of up to 3 mM, but they were more acid reistant than the *L. paracasei* strains when challenged with 1M HCl (pH 2.5) with an average of 3 log reduction of CFU/ml. They were also sensitive to 30 µg tetracycline, 10 µg ampicillin, 15 µg erythromycin, 30 µg chloramphenicol, but similarly to reference strains showed intrinsic resistance to 30 µg vancomycin, 5 µg ciprofloxacin and 30 µg kanamycin. *L. rhamnosus and L. plantarum* on the other hand showed low GABA production and were resistant to 10 µg gentamycin in 17 out of 18 strains (94%). *L. paracasei* showed intermediate susceptibility to 10 µg gentamycin in 15 out of 19 strains (78%). Furthermore, from the results of antimicrobial activity, *L. rhamnosus* showed no inhibition against *Listeria monocytogenes* while *L. plantarum* and *L. paracasei* exhibited minor inhibition. As a result, there is no significant difference ($p<0.05$) between the species in terms of diameter of inhibition zones when testing for antimicrobial activity against *List. monocytogenes*. Additionally, the ability to form biofilm was assessed and 15 out of 18 *L. plantarum* strains were determined as strong biofilm producers based on their ability to form biofilm of up to optical density of 7 at 595 nm wavelength while *L. paracasei* and *L. rhamnosus* were weak biofilm producers. Furthermore, strains were assessed for their ability to acidify, *L. paracasei* species could attain an average (between all isolates) of pH 4.6 after 24 h and an average pH of 3.9 at 48 h, while *L. plantarum*

reached an average pH of 5.2 at 24 h and of 4.7 at 48 h and *L. rhamnosus* a pH of 5.1 at 24 h and a pH of 4.5 at 48 h respectively. Finally, all the strains showed a 0.15% minimum inhibitory concentration (MIC) to bile salts (Sodium deoxocholate). Furthermore, we performed batch culture fermentation experiments where the vessels were inoculated with a suspension of approximately $1\times10^8$ cfu/ml of *L. plantarum* (M77 & M84), two major GABA producers (GABA levels > 2.5 mM). There was a non-significant ($p>0.05$) increase in GABA production at 12 h and 48 h fermentation comparing to the control. Furthermore, there was also non-significant ($p<0.05$) increase in acetate, butyrate and propionate production following fermentation with *L. plantarum* M77 and M84 at 24 h and 48 h respectively.

# IMPORTANCE

Lactic acid bacteria (LABs) are the most important group of probiotics and starter cultures. The consumption of LABs, provide several health benefits to humans as well as food processing technology. Although fermented products are well-known as good sources of LABs, however, the human gut microflora is also considered as a natural habitat of LABs. Therefore, before a bacterium can be classed as probiotic, it needs to be tested for its safety and also its ability to withstand technological processes. The importance of our research is to investigate probiotic properties of GABA producing LAB strains isolated from human gut which could be used to develop new probiotics as well as starter cultures for fermented food products that could elicit positive health effects associated with GABA.

# 1. Introduction

Lilly and Stillwell (1965) were the first to use the term probiotics meaning 'for life' and it was used to describe substances produced by a microorganism to stimulate the growth of other organisms. A general, and acceptable definition for probiotics was then given according to FAO/WHO (2001) and they are defined as "live microorganisms which when consumed in adequate amounts confer a health effect on the host''. In 2014, this definition was updated in 2014 by the International Scientific Association for Probiotics and Prebiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014).

Lactic acid bacteria are normally used as probiotics and species belonging to the *Lactococcus* and *Lactobacillus* genera are mostly used because they are generally regarded as safe (GRAS). They have also been used as they are believed to have desirable effects on the host and also they have been widely used by the dairy and pharmaceutical industries for the production of functional foods (Collado et al., 2007, Salminen et al., 1998, Tannock, 1997). LABs have been widely used in food production as probiotics, starter cultures, non-starter and/or adjuncts starter. LAB strains with low acidifying capacity can be used as adjunct starter cultures alongside the primary starter cultures (responsible for acidification) in order to enhance the flavour, texture or aroma of the product (Akabanda et al., 2014).

Probiotics influences the composition and function of the intestinal microbiome by reducing the growth of a pathogen by producing antimicrobial compounds such as bacteriocins, ethanol, organic acids and hydrogen peroxide (Pisano et al., 2014).

The use and beneficial effect of probiotics has been widely studied while some of these health benefits have been established while others have shown great potential in animal models, while more human clinical trials are required to confirm these claims (Fuller, 1989, Argyri et al., 2013). Some of the beneficial effects of probiotics are usually associated with its potential

therapeutic effect on gastrointestinal disorders such as diarrhoea, constipation, irritable bowel syndrome (Rolfe, 2000). In addition, probiotics have been shown to stimulate the immune system, enhance bowel movement and lower cholesterol levels (Ashraf and Shah, 2014, Whelan and Quigley, 2013, Choi and Chang, 2015, McFarland, 2007). With all these health benefits it should be noted that different strains of bacteria expresses their effect differently on the host i.e. there is no strain that can give all the health benefits at once (Vasiljevic and Shah, 2008). It is also very important to note that as probiotics, the bacteria should be viable by the time they reach the intestinal tract in order to colonise the intestinal tract and benefit the host (Hyronimus et al., 2000).

Research conducted on the use of probiotics has shown various evidence in improving the host health i.e. health claims on microorganisms defined as probiotics suggests a link between the specific food and maintaining good  that the food can reduce the risk of some diseases (van Loveren et al., 2012). The European Food safety Authority (EFSA) have published health claims aimed at consumer protection which focuses on using the term probiotics in food or food labels, and assessing its relationship to the host health and also assessing studies and giving priority to controlled human intervention studies (EFSA, 2011). The limitations of this studies are linked to the fact that intervention studies using probiotics are conducted on subjects with risk factors to many diseases rather than healthy subjects which makes it difficult in validating the claims. Therefore, EFSA has suggested according to available assessments a need for further research work based on probiotics (Salminen and van Loveren, 2012).

Lately, many studies have focused on the positive health effects of γ-aminobutyric acid (GABA), which is a compound produced by many microorganisms including many LABs. Based on the above, many companies produce GABA-enriched foods and many studies view GABA as a desirable property falling under the category probiotic properties. In the present

study we looked into various probiotic properties of various GABA-producing LAB strains isolated from the human gut as probiotics.

GABA is the main inhibitory neurotransmitter in the central nervous system of mammals and it acts by binding to either the $GABA_A$ or $GABA_B$ receptors (Li et al., 2010, Dhakal et al., 2012, Wang and Kriegstein, 2009). It is a non-protein amino acid that is widely distributed in nature i.e. it is found in plants, mammals and some insects and although it is not a part of the Krebs cycle it affects it as GABA is metabolised by transamination with α-ketoglutarate to produce glutamate and succinic semi-aldehyde which is then oxidised to succinate that goes into the Krebs cycle. (Diana et al., 2014).

The production of GABA unlike other neurotransmitters has been widely studied for its physiological and psychological functions in both human and animal models such as blood pressure in both hypertensive rats and human intervention trials (Hayakawa et al., 2004, Inoue et al., 2003, Tanaka et al., 2009). In addition, GABA has been linked with various diseases such as Parkinson's, seizures, Alzheimer's (Ting Wong et al., 2003, Battaglioli et al., 2003), prevention of sleeping disorders, mood disorders, and depression (Ting Wong et al., 2003, Sasaki et al., 2006, Krystal et al., 2002, Bjork et al., 2001). Furthermore, GABA has been shown to slow down the proliferation of various cancer cells, and it is considered to help in suppressing tumours (Schuller et al., 2008, Oh and Oh, 2004, Kleinrok et al., 1998) .

With all the health benefits of GABA it is important to study the potential use of GABA-producing LAB strains for their probiotic properties in order to be used in functional foods production and some of the criteria used to select a strain for its probiotic potential include origin i.e. it must be of human origin, have the ability to survive during gastric passage, be bile tolerant, acid resistant, antibiotic sensitive, able to adhere to the gut epithelial tissue and genetically stable (Vasiljevic and Shah, 2008, Morelli, 2007).

Bearing all this in mind we decided to study 38 LAB strains alongside two reference strains for various probiotic properties, including GABA production and then based on the results, two major GABA-producing strains were selected to assess their ability to affect GABA and short chain fatty acid (SCFA) production in batch culture fermentation and also they had their genome sequenced.

# 2. Materials and Methods

### 2.1 Isolation and identification of LAB strains

Ten percent (w/v) of faecal slurry was prepared by taking fresh faecal samples from a healthy volunteer (without antibiotics for 6 months) mixed with the stomacher for 2 min in phosphate saline buffer (PBS, pH 7.4). Decimal dilutions of the sample were prepared and 100 µl of each dilution were spread on de Man-Rogosa and Sharp agar (MRS; Sigma-Aldrich, Dorset, UK) and incubated anaerobically at 37 $^{o}$C for 48 h. Colonies were selected from appropriate dilution and purified on MRS agar and also incubated anaerobically at 37 $^{o}$C for 48 h. Pure cultures were stored at -80 $^{o}$C in cryovial tubes containing MRS broth with the addition of 7% (vol/vol) Dimethyl sulphoxide (DMSO; Sigma-Aldrich, Dorset, UK). Each colony was sub-cultured, selected and identified based on their catalase activity using hydrogen peroxide, on being Gram positive during the Gram staining technique and also biochemically using the API 50 CH identification kit along with API 50 CHL medium (Biomerieux,UK).

#### 2.1.1 Identification on API Fermentation kit:

API 50 CHL Kit (50410-bioMérieux, UK) was used for the identification of the isolates. Frozen strains were subcultured in MRS broth at 37 $^{o}$C for 24 h before isolation on MRS agar for 48h at 37 $^{o}$C. The purity of the strains was verified by microscopic examination. API 50 CH Strips were set in the incubation box and covered with the lid. An ampule of API Suspension Medium

(2ml) was used to make a heavy bacterial suspension of the bacteria from the culture using a sterile swab. Then, a suspension with a turbidity equivalent to 2 McFarland standard was prepared by transferring 2-5 drops of the bacterial suspension into the ampule of API Suspension Medium (5 ml). An ampule of API 50 CHL Medium inoculated by transferring some of the bacterial suspension into the ampule and was used immediately. The tubes of the strip were filled with the inoculated API 50 CHL Medium and covered with mineral oil. The strips were incubated aerobically at 37 $^{o}$C for 48 h and results were recorded on the result sheets at both 24 h and 48 h. The biochemical profile obtained for each strain was identified using the *apiweb*$^{TM}$ identification software with database (V5.1).

**2.2 Bacterial cultures, growth conditions and media**

In this study, 38 human gut LAB isolates including *Lactobacillus plantarum* (18), *Lactobacillus paracasei* (19), *Lactobacillus rhamnosus* (1), human skin *Listeria monocytogenes* and reference strains *L. rhamnosus GG* and *L. casei Shirota* isolate was used. Human gut LAB strains were selected based on their acid and bile resistance and GABA production capacity in the previous study. Stock cultures were maintained at -80 °C in 7% (vol/vol) DMSO (Sigma-Aldrich, Dorset, UK). Before experiments, LAB agar-plate cultures were prepared from stock cultures by streaking onto MRS agar (Sigma Aldrich. Lancashire, UK) and incubated for 48 h at 37°C in an anaerobic incubator (Don Whitley Scientific,Bingley,UK) with 80:10:10 $N_2$:$CO_2$:$H_2$ gas ratio. After incubation, MRS agar cultures were stored at 4 °C to be used in further experiments. Throughout the study, fresh culture agar plates were prepared every two weeks.

Before every experiment, in order to prepare overnight MRS broth cultures, 3 colonies from MRS agar plates stored at 4 °C were inoculated into 3 ml sterile MRS broth in Sterilin

Quickstart Universal polystyrene 7 mL containers (Thermo Scientific, Loughborough, UK) and incubated at 37 °C in an anaerobic incubator.

Before experiments, *List. monocytogenes* agar-plate cultures were prepared from stock cultures by streaking onto brain heart infusion broth (BHI; LAB M, Lancashire, UK) and incubated for 24 h at 37 °C in an aerobic incubator. After incubation, BHI agar cultures were stored at 4 °C to use for antimicrobial assay.

To prepare overnight broth cultures, 3 colonies were transferred from BHI agar (LAB M, Lancashire, UK) culture into 3 ml sterile BHI broth and incubated at 37 °C with shaking (160 rpm) for 8 h. Then, in order to increase the volume, 10 µl BHI broth culture were transferred into 10 ml BHI broth (1% vol/vol) and incubated overnight at 37 °C with shaking (160 rpm).

### 2.2 Survival in simulated gastric conditions

Following isolation and identification, the strains were tested for their probiotic properties and survival in simulated gastric conditions with all tests carried out in triplicate. The tests carried out were for acid resistance, bile resistance, antibiotic resistance, anti-listerial activity and biofilm formation. The methods used were modified according to (Argyri et al., 2013), (Balamurugan et al., 2014), and (Pavli et al., 2016). All compounds were purchased from Sigma Aldrich (Dorset, UK) except otherwise stated.

#### 2.2.1 Acid resistance

The strains (3 colonies in 3ml of broth) were cultured anaerobically at 37 $^{o}$C for 24 h in MRS broth. Overnight cultures (1ml) were then centrifuged (11337 ×g for 10 min) and pellets were re-suspended in MRS broth and the pH was adjusted to 2.5 using 1M HCl. The bacterial cells were incubated anaerobically at 37 $^{o}$C for 1 h. After 1 h the sample culture were serially diluted,

plated on MRS agar and inoculated at 37 $^{o}$C for 24 h. Survival was assessed through colony counts after 24 h on MRS agar plates in triplicate and the reduction in counts were calculated.

### 2.2.2 Bile resistance

Overnight (24 h) cultures of bacterial cells were harvested by centrifugation (11337 ×g for 10 min) and subsequently the pellets were re-suspended in MRS broth containing 0.02 % or, 0.04 % or, 0.08 % or, 0.15 % or, 0.3 % or, 0.6 % w/v sodium deoxocholate (bile salt). The resistance to sodium deoxocholate was assessed by incubating and growing the strains in 96-well plates to determine the Minimum Inhibitory Concentration (MIC) using a plate reader (Sunrise, TECAN, Mannedorf, Switzerland). The plate reader was run at wavelength of 625 nm at 37$^{o}$C for 48 h and data was collected using Magellan software (TECAN, Mannedorf, Switzerland). The readings were taken at 20 min intervals and results were analysed using Microsoft Excel software.

### 2.2.3 Antibiotic resistance

The disc diffusion method of Kirby-Bauer method ((Sandle, 2016) was used in which an overnight suspension of approximately $10^{8}$ CFU/ml was prepared according to 0.5 McFarland standard and then 100 µl of the isolates were spread on MRS agar plates and different antibiotic discs (Oxoid, UK) with defined concentrations (vancomycin 30 µg, streptomycin 25 µg, ciprofloxacin 5 µg, tetracyclin 30 µg, ampicillin 10 µg, kanamycin 30 µg, chloramphenicol 30 µg, erythromycin 15 µg, and gentamicin 10 µg) Thermo Scientific; Oxoid, Basingstoke, UK were implanted in the inoculated MRS plates and incubated anaerobically at 37$^{o}$C for 24 h. After incubation, the growth inhibition zones around each disc were measured and results recorded as susceptible or resistance to the antibiotics based on comparison with standards presented in the literature and results were analysed using Microsoft Excel software.

### 2.2.4 Antimicrobial activity against *Listeria monocytogenes*

This method was modified according to (Harris et al., 1989). *L. monocytogenes* 10403S WT strain was used as the indicator organism and it was subcultured in BHI broth (Lab M, Lancanshire UK) at 37 $^{o}$C for 24 h. The Lactic acid bacteria strains were also cultured for 24 h in MRS broth (Sigma, Dorset, UK). Two hundred µl of the *L. monocytogenes* strain was spread onto BHI agar plates. Paper discs were prepared by punching Whatmann 21 mm glass microfiber filters in 6 mm diameter and then were discs wrapped in foil and autoclaved. Overnight broth cultures of LAB were filtered through Minisart filter with 0.2 µm pore size and then 10 µl cell-free filtrate was embedded in the sterile discs. The LAB embedded discs were impregnated in the BHI plates inoculated with *L. monocytogenes* and incubated at 37 $^{o}$C for 24 h. After incubation, the growth inhibition zones around each disc were measured and results recorded and analysed using Microsoft Excel software.

### 2.2.5 Acidification

The acidification ability of a lactic acid bacterium is very important as it can safeguard fermented products through low pH. The acidification was assessed according to (Akabanda et al., 2014) with minor modifications. Overnight MRS broth cultures of 38 lactic acid bacteria was inoculated 1% (vol/vol) in 3 mL semi-skimmed pasteurised milk with 1.5% fat content in Sterilin Quickstart Universal polystyrene 7 mL containers (Thermo Scientific, Loughborough, UK) and were incubated anaerobically at 37 °C. Throughout the incubation period, the pH changes were measured with a pH meter (Mettler Toledo, Columbus, Ohio, USA) at 0, 24 and 48 h.

### 2.2.6 Biofilm formation

Biofilm formation was assessed according to (O'Toole, 2011, Fernandez Ramirez et al., 2015). One micro-liter of each LAB strain was inoculated in 100 µl MRS broth and cultured anaerobically at 37ºC for 24 h in 96-well microtiter plates (Greiner Bio-One; UK). Subsequently, 2 µl from the latter overnight cultures were transferred into 96-well plates, and added 198 µl MRS broth (1%) and incubated at 37 °C for 48 h. For control samples, 3 mL sterile MRS broth was used and exposed to the same procedure. The crystal violet assay was used in determining and quantifying the biofilm formation. After incubation, the supernatant was carefully removed from the wells together with the planktonic cells using micropipette and the biofilm formed was washed thrice using 200 µl sterile phosphate saline buffer (PBS). The remaining biofilm was then stained using 200 µl of 0.1 % crystal violet and left to stand for 30 min and the excess crystal violet was carefully removed and washed again thrice using PBS. The dye attached to the biofilm was then solubilised in 70 % ethanol for 30 min and the optical density (OD) was measured at 590 nm using plate reader (BMG Labtech; Offenburg, Germany).

### 2.2.7 γ-aminobutyric acid production

To assess the γ-aminobutyrate production we used the method of (Tsukatani et al., 2005) modified by (Karatzas et al., 2010). In short, strains were grown in MRS broth (3 colonies in 3 ml of broth) overnight under anaerobic conditions and then used as inoculum for a second culturing in MRS broth (50 µl of culture into 5 ml of broth containing 10 mM glutamate) for 48 h, 500 µl of the latter culture centrifuged at 11337 ×g for 10 min. The supernatants were stored in Eppendorf tubes and kept frozen until they were needed for the assay. A GABAse master mix was prepared containing 80 mM Tris (base) amino methane, 2 mM α-ketoglutarate,750 mM sodium sulphate ($Na_2SO_4$ salt), 10 mM dithiothreitol (DTT), 1.4 mM beta-nicotinamide adenine dinucleotide phosphate (NADP) and 0.3 g/l GABAse enzyme from

*Pseudomonas fluorescens* (Sigma Aldrich, Dorset, UK) in sterile water was prepared. 90 µl of the master mix was placed in the wells of a 96-well plate and subsequently, 10 µl of the defrosted supernatant was added. GABA standard solutions containing a known amount of GABA (0, 2, 4, 6, 8 and 10 mM) were prepared and run alongside the samples. The plate was run at wavelength of 340 nm at 37 $^{o}$C for 3 h using the Sunrise plate reader and data was collected using Magellan software (TECAN, Mannedorf Switzerland). The readings were taken at intervals of 2 min and the results were analysed using Microsoft Excel software.

### 2.2.8 Batch culture fermentation (non-pH controlled)

The batch culture fermentation was done according to the method described by (Olano-Martin et al., 2000). Basal nutrient medium containing peptone water, 2 g/l peptone water, 2 g/l yeast extract (Oxoid, Hampshire, UK), 0.1 g/lNaCl, 0.04 g/l $K_2HPO_4$, 0.04 g/l $KH_2PO_4$, 0.01 g/l $MgSO_4.7H_2O$ (Fischer scientific, Loughborough, UK), 0.01 g/l $CaCl_2.6H_2O$, 2 g/l $NaHCO_3$ (Fischer scientific, Loughborough, UK), 0.05 g/l hemin (dissolved in a few drops of 1 mol $l^{-1}$ NaOH), 0.5 g/l cysteine.HCl, 0.5 g/l bile salts , 2 g/l Tween 80, 4 ml resazurin (0.025g/100ml, pH7) and 10 µl vitamin $K_1$ was autoclaved and 8 ml was dispensed in Hungate tubes and placed in the anaerobic cabinet overnight at 37 $^{o}$C to make it anaerobic before inoculation. The compounds used were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated.

Then a suspension of approximately $10^8$ CFU/ml of the overnight *L. plantarum* culture was prepared according to 0.5 McFarland standard. In addition, 10 % (w/v) of faecal slurry was prepared by taking fresh faecal samples from five healthy volunteers (not receiving antibiotics for 6 months) mixed with the stomacher for 2 min in phosphate saline buffer.

Then 1 ml of the suspension was inoculated with 1 ml of faecal slurry in 8 ml of the overnight basal medium (in Hungate tubes). One ml of each of the samples was then taken at 0, 12, 24, & 48 h for GABAse analysis, SCFA analysis and sequencing.

### 2.2.9 Short chain fatty acid (SCFA) analysis

The SCFAs in faecal samples was measured using gas chromatography (GC) flame ionisation detection and the derivatisation was performed according to (Richardson et al., 1989) with modifications.

Fifty µl of 0.1 M 2-ethylbutyric acid solution was added to 1 ml of faecal sample in Hungate tubes and the vortexed. The mixture was acidified with 0.5 ml concentrated HCl before adding 2 ml of diethyl ether. The mixture was properly vortexed and then centrifuged at 1700 ×g for 10 min. Zero point four ml of diethyl ether extract and 20 µl derivatising reagent N-tert-butyldimethylsilylN-methyltrifluoroacetamide (MTBSTFA) containing 1% tetrabutyldimethylcholorosilane (1% TBDMSCI; Sigma-Aldrich, Auckland, New Zealand) were placed into a 2 ml glass vial and capped. The mixtures were incubated at room temperature for 72 h to ensure that derivatisation was complete. Standard mixtures containing 5 mM, 2-ethyl butyrate for calibration were extracted and derivatised using the same steps as these of the faecal samples. The samples and standards were analysed using the GC.

Analysis was performed using an Agilent/HP 6890 series GC (Hewlett Packard, UK) equipped with a flame ionization detector and an Agilent HP-5MS 30 m × 0.25 mm column with a 0.25 µm coating of cross-linked (5%-Phenyl)-methylpolysiloxane, (Hewlett Packard, UK). The carrier gas which is helium has a flow rate of 1.7 ml $min^{-1}$ and head pressure of 113 kPa. The initial temperature is 63 $^{o}C$ which was elevated by 15 $^{o}C$ $min^{-1}$ till it reached 190 $^{o}C$ and then it was held for 3 min. The injector and detector temperatures were set at 275 $^{o}C$. The test samples were injected (1µl) with a split ratio of 100:1. The instrument was monitored, and the chromatograms were acquired using GC offline software.

### 2.2.10 DNA Isolation for genomic sequencing

DNA isolation was performed according to (Abed, 2013) with minor modifications. The wizard genomic DNA purification kit (Promega, USA) was used for this purpose. The cells were grown in MRS containing 1.5 % glycine to aid cell lysis. Five ml of the culture was centrifuged at 11337×g for 3 min and the pellets re-suspended in 900 µl of 50 mM EDTA. One hundred twenty µl lysozyme (40 mg/ml, Sigma Aldrich, Dorset, UK) was added to the cell suspension and incubated in water bath at 37 $^{o}$C for 1 h with occasional mixing. The suspension was centrifuged at 11337×g for 3 min and the pellets re-suspended in 900 µl of nuclei lysis solution (Promega, Wisconsin, U.S.A). The cells were then incubated at 80 $^{o}$C for 5 min to induce lysis and then cooled at room temperature. The 4 µl of RNase solution (50 mg/ml. Sigma Aldrich, UK) was added to the tubes, the mixture was gently vortexed and then incubated at 37 $^{o}$C for 1 h. For protein precipitation, 300 µl of the precipitation solution (Promega, Wisconsin,U.S.A) was added to the mixture and vigorously vortexed for 30 sec, then the mixture was incubated on ice for 7 min and further centrifuged at 11337×g for 15 min. The supernatant containing the DNA was transferred to a clean 1.5 ml micro centrifuge tube containing 600 µl of room temperature isopropanol. The mixture was gently mixed by inversion until threadlike strands of DNA formed a visible mass and the mixture was centrifuged at 11337×g for 10 min. The pellet was gently washed with 70 % ethanol to remove residual contaminant then the mixture was centrifuged at 11337×g for 10 min. The ethanol was carefully aspirated and the pellet was air dried for 15 min. The pellet was re-suspended in 60 µl of the Nuclease free water (Thermo Scientific, UK) to re-hydrate the DNA. The DNA was rehydrated overnight by gently shaking at 25 $^{o}$C. The DNA was then stored at -20 $^{o}$C which was later sent to MicrobesNG, Birmingham, UK for full genome sequencing.

### 2.2.11 Whole genome sequencing (MicrobesNG)

Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer. DNA was quantified in triplicate with the Quantit dsDNA HS assay in an Ependorff AF2200 plate reader. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: 2 ng of DNA instead of 1 were used as input, and PCR elongation time was increased to 1 min from 30 s. DNA quantification and library preparation were carried out on a Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol.

Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 (Bolger et al., 2014). De novo assembly was performed on samples using SPAdes version 3.7 (Bankevich et al., 2012), and contigs were annotated using Prokka 1.11 (Seemann, 2014)

## 2.3 Statistical Analysis

Statistical results were given based on paired samples T-test and one-way ANOVA and $p<0.05$ indicated that the results were statistically significant. The statistical analysis for acid resistance was based on Pearson correlation using SPSS. For GABA production, T-test was performed using excel and Kraken software which were used to analyse the whole genome sequencing data (performed by Microbes NG, UK).

# 3. Results

## 3.1 Identification of LAB strains using Biomereux API 50CH identification kit and software

The identification of the strains was performed using the Biomereux API 50CH identification kit and identification at the species level was based on their ability to detect enzymatic activities usually related to metabolising various carbohydrates, catabolism of proteins or amino acids by the tested organisms. The biochemical profile of the isolates was analysed using apiwebTM identification software with database (V5.1). Sixteen out of the 18 strains were identified by 99 % as *L. plantarum* while the remaining 2 where identified as such by 96 %. All remaining strains were identified by 99 % as *L. paracasei* and *L. rhamnosus*.

**Table 1:** Identification of LAB strains using the API 50CH identification kit.

| Code | Strains | Identity (%) |
|---|---|---|
| M92 | *L. plantarum* | 99.9 |
| M93 | *L. plantarum* | 99.9 |
| M88 | *L. plantarum* | 99.9 |
| M89 | *L. plantarum* | 99.9 |
| M87 | *L. plantarum* | 99.9 |
| M86 | *L. plantarum* | 99.9 |
| M84 | *L. plantarum* | 99.9 |
| M85 | *L. plantarum* | 99.9 |
| M64 | *L. paracasei* | 99.8 |
| M63 | *L. paracasei* | 99.9 |
| M57 | *L. paracasei* | 99.8 |
| M38 | *L. paracasei* | 99.8 |
| M35 | *L. paracasei* | 99.9 |
| M77 | *L. plantarum* | 99.9 |
| M75 | *L. plantarum* | 96.7 |
| M73 | *L. plantarum* | 99.9 |
| M70 | *L. paracasei* | 99.9 |

| | | |
|---|---|---|
| M69 | *L. paracasei* | 99.6 |
| M66 | *L. paracasei* | 99.9 |
| M65 | *L. paracasei* | 99.9 |
| M59 | *L. paracasei* | 99.8 |
| M96 | *L. plantarum* | 96.7 |
| M95 | *L. plantarum* | 99.9 |
| M56 | *L. paracasei* | 99.8 |
| M47 | *L. paracasei* | 99.6 |
| M44 | *L. rhamnosus* | 99.9 |
| M41 | *L. paracasei* | 99.9 |
| M40 | *L. paracasei* | 99.8 |
| M39 | *L. paracasei* | 99.9 |
| M36 | *L. paracasei* | 99.9 |
| M22 | *L. plantarum* | 99.9 |
| M94 | *L. plantarum* | 99.9 |
| M79 | *L. plantarum* | 99.9 |
| M19 | *L. paracasei* | 99.6 |

### 3.2 Whole genome sequencing

The whole genome sequencing was carried as an alternative to the API 50CH identification kit and more accurate method of identification of the strains. The two strains we preformed genome sequencing were identified as *Lactobacillus plantarum* species with M84 having an identity of 84.64 % and M77 giving 81.55 % identity (Table 2).

**Table 2:** Top families and genera that the reads map to, this has been calculated using the software kraken.

| Sample | Most frequent family | Percentage (%) | Most frequent genus | Percentage (%) | Most frequent species | Percentage (%) |
|---|---|---|---|---|---|---|
| **M84** | *Lactobacillaceae* | 89.24 | *Lactobacillus* | 88.89 | *Lactobacillus plantarum* | 84.64 |
| **M77** | *Lactobacillaceae* | 86.86 | *Lactobacillus* | 86.34 | *Lactobacillus plantarum* | 81.55 |

### 3.3 Determination of acid resistance of isolates.

The resistance of each strain in 1M HCl for 1 h at pH 2.5 was determined. *L. plantarum* strains showed higher acid resistance with 13 out of the 18 (≃ 72 %) strains having about 3 log reduction of CFU/ml while *L. paracasei* were more sensitive with 11 out of the 19 (≃ 58 %) strains demonstrating more than 4 log (CFU/ml) reduction. The *L. rhamnosus* strain showed a significant acid resistance having 1 log reduction of CFU/ml. While the *L.rhamnosus* 9A and *L. casei shirota* 11A strains were used as reference strains.

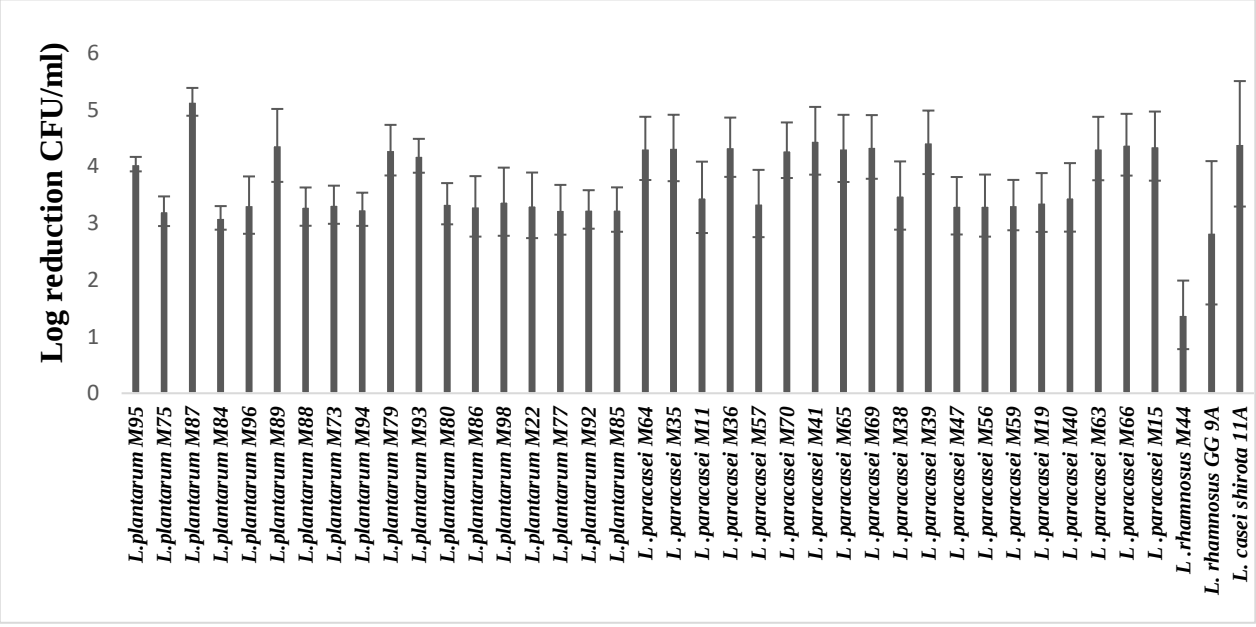



**Fig 1:** Log reduction in CFU/ml of LAB isolates at pH 2.5 for 1 h (adjusted with 1M HCl).

### 3.4 Determination of antimicrobial activity against *Listeria monocytogenes.*

In the disc diffusion assay set up, the anti-microbial activity of 38 human gut LAB isolates was determined. From the 18 *L. plantarum* strains, 17 strains (≃ 95 %) exhibited a growth inhibition zone with a diameter ranging between 6 and 8.3 mm against *List. monocytogenes.* Only 8 *L. paracasei* strains (≃ 42%) showed an inhibitory zone in a range from 6 to 8.5 mm while *L. rhamnosus* showed no inhibition against *List. monocytogenes*. Which means that there is little or no inhibition as the diameter of the disc is 6mm.

**Table 3:** Antimicrobial inhibitory activity of 38 human gut LAB strains against growth of *List. monocytogenes* as indicated by inhibition zones (diameters in mm) following disc diffusion test

| Strains | Inhbition Zone Diameter (mm) | Diameter showing minor/no inhibition(mm) i.e. diameter of inhibition zone – diameter of disc (6 mm) |
|---|---|---|
| *L. plantarum* M95 | 7.2 | 1.2 |
| *L. plantarum* M75 | 6.8 | 0.8 |
| *L. plantarum* M87 | 6.7 | 0.7 |
| *L. plantarum* M84 | 7.7 | 1.7 |
| *L. plantarum* M96 | 6.8 | 0.8 |
| *L. plantarum* M89 | 6.0 | 0 |
| *L. plantarum* M88 | 6.1 | 0.1 |
| *L. plantarum* M73 | 7.3 | 1.3 |
| *L. plantarum* M94 | 8.3 | 2.3 |
| *L. plantarum* M79 | 7.1 | 1.1 |
| *L. plantarum* M93 | 7.5 | 1.5 |
| *L. plantarum* M80 | 6.2 | 0.2 |
| *L. plantarum* M86 | 6.2 | 0.2 |
| *L. plantarum* M98 | 6.3 | 0.3 |
| *L. plantarum* M22 | 6.2 | 0.2 |
| *L. plantarum* M77 | 8.2 | 2.2 |
| *L. plantarum* M92 | 6.8 | 0.8 |
| *L. plantarum* M85 | 6.7 | 0.7 |
| *L. paracasei* M64 | 6.0 | 0 |
| *L. paracasei* M35 | 6.7 | 0.7 |
| *L. paracasei* M11 | 6.0 | 0 |
| *L. paracasei* M36 | 6.0 | 0 |
| *L. paracasei* M57 | 7.3 | 1.3 |
| *L. paracasei* M70 | 6.0 | 0 |
| *L. paracasei* M41 | 6.0 | 0 |
| *L. paracasei* M65 | 7.4 | 1.4 |
| *L. paracasei* M69 | 7.7 | 1.7 |
| *L. paracasei* M38 | 6.0 | 0 |
| *L. paracasei* M39 | 6.0 | 0 |
| *L. paracasei* M47 | 8.5 | 2.5 |
| *L. paracasei* M56 | 6.0 | 0 |
| *L. paracasei* M59 | 6.0 | 0 |
| *L. paracasei* M19 | 6.0 | 0 |
| *L. paracasei* M40 | 8.1 | 2.1 |
| *L. paracasei* M63 | 6.0 | 0 |
| *L. paracasei* M66 | 8.0 | 2.0 |
| *L. paracasei* M15 | 7.0 | 1.0 |
| *L. rhamnosus*M44 | 6.0 | 0 |

**Table 4:** Classification of LAB species based on antimicrobial activity against *L. monocytogenes* by disc diffusion methods.

| **Parameters** | ***L. plantarum*** | ***L. paracasei*** | ***L. rhamnosus*** |
|---|---|---|---|
| **Mean diameter of inhibiton zone (mm)** | 6.9 | 6.7 | 6 |
| **Inhibition zone range (mm)** | 6 – 8.3 | 6 – 8.5 | 6 |
| **Strains with no effect (%)** | 55 | 57.9 | 100 |

### 3.5 Antibiotic resistance

Antibiotic susceptibility of LAB strains was divided into 3 groups; resistant, intermediate and susceptible and are considered resistant when they showed MIC values above the MIC breakpoints established by the European Food Safety Authority (EFSA, 2008) against 8 antibiotics with different concentrations based on the zone of inhibition (Table 5). All LAB strains were found to be resistant to vancomycin, ciprofloxacin, chloramphenicol and kanamycin whereas they were susceptible to kanamycin, erythromycin, tetracycline and ampicillin except *L. plantarum* M85 which showed intermediate susceptibility against chloramphenicol and *L. paracasei* strains M35, M36, M65 and M63 which showed intermediate susceptibility to gentamycin (Table 6). *L. rhamnosus GG* and *L. casei Shirota* were used as reference strains. The strains have similar antibiotic profile/charcteristics with the *L. casei Shirota* (table 5).

**Table 5:** Inhibition zone diameters (mm) of human gut LAB strains against eight antibiotics by disc diffusion antibiotic susceptibility test

| | | Inhibitory Zone Diameter (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Species** | **Strains** | **VA** | **CIP** | **TE** | **AMP** | **CN** | **K** | **C** | **E** |
| ***L. plantarum*** | M95 | 6(r) | 6(r) | 26(s) | 41(s) | 7(r) | 6(r) | 30(s) | 27(s) |
| | M75 | 6(r) | 6(r) | 23(s) | 35(s) | 8(r) | 6(r) | 30(s) | 26(s) |
| | M87 | 6(r) | 6(r) | 22(s) | 37(s) | 7(r) | 6(r) | 30(s) | 27(s) |
| | M84 | 6(r) | 6(r) | 24(s) | 38(s) | 7(r) | 6(r) | 30(s) | 25(s) |
| | M96 | 6(r) | 6(r) | 24(s) | 37(s) | 8(r) | 6(r) | 30(s) | 25(s) |
| | M89 | 6(r) | 6(r) | 23(s) | 39(s) | 8(r) | 6(r) | 31(s) | 26(s) |
| | M88 | 6(r) | 6(r) | 24(s) | 38(s) | 8(r) | 6(r) | 29(s) | 26(s) |
| | M73 | 6(r) | 6(r) | 32(s) | 36(s) | 11(r) | 6(r) | 28(s) | 33(s) |
| | M94 | 6(r) | 6(r) | 23(s) | 39(s) | 9(r) | 6(r) | 31(s) | 27(s) |
| | M79 | 6(r) | 6(r) | 25(s) | 38(s) | 7(r) | 6(r) | 31(s) | 25(s) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | M93 | 6(r) | 6(r) | 25(s) | 37(s) | 8(r) | 6(r) | 32(s) | 27(s) |
| | M80 | 6(r) | 6(r) | 23(s) | 33(s) | 7(r) | 6(r) | 27(s) | 24(s) |
| | M86 | 6(r) | 6(r) | 23(s) | 39(s) | 7(r) | 6(r) | 29(s) | 26(s) |
| | M98 | 6(r) | 6(r) | 21(s) | 35(s) | 7(r) | 6(r) | 28(s) | 24(s) |
| | M22 | 6(r) | 6(r) | 22(s) | 37(s) | 7(r) | 6(r) | 29(s) | 29(s) |
| | M77 | 6(r) | 6(r) | 24(s) | 37(s) | 7(r) | 6(r) | 30(s) | 25(s) |
| | M92 | 6(r) | 6(r) | 25(s) | 36(s) | 6(r) | 6(r) | 31(s) | 25(s) |
| | M85 | 6(r) | 6(r) | 24(s) | 38(s) | 13(i) | 6(r) | 31(s) | 27(s) |
| ***L. paracasei*** | M64 | 6(r) | 8(r) | 42(s) | 33(s) | 12(i) | 6(r) | 35(s) | 34(s) |
| | M35 | 6(r) | 11(r) | 42(s) | 33(s) | 11(r) | 6(r) | 33(s) | 34(s) |
| | M11 | 6(r) | 11(r) | 42(s) | 36(s) | 14(i) | 6(r) | 38(s) | 39(s) |
| | M36 | 6(r) | 13(r) | 39(s) | 36(s) | 9(r) | 6(r) | 38(s) | 30(s) |
| | M57 | 6(r) | 8(r) | 33(s) | 35(s) | 13(i) | 6(r) | 34(s) | 34(s) |
| | M70 | 6(r) | 14(r) | 43(s) | 37(s) | 13(i) | 6(r) | 37(s) | 34(s) |
| | M41 | 7(r) | 9(r) | 38(s) | 34(s) | 13(i) | 6(r) | 34(s) | 37(s) |
| | M65 | 6(r) | 9(r) | 38(s) | 37(s) | 6(r) | 6(r) | 37(s) | 35(s) |
| | M69 | 7(r) | 11(r) | 44(s) | 33(s) | 13(i) | 6(r) | 40(s) | 38(s) |
| | M38 | 6(r) | 9(r) | 42(s) | 36(s) | 14(i) | 6(r) | 36(s) | 34(s) |
| | M39 | 6(r) | 14(r) | 44(s) | 37(s) | 13(i) | 6(r) | 37(s) | 36(s) |
| | M47 | 6(r) | 9(r) | 35(s) | 33(s) | 14(i) | 6(r) | 35(s) | 33(s) |
| | M56 | 6(r) | 14(r) | 46(s) | 37(s) | 12(i) | 6(r) | 40(s) | 37(s) |
| | M59 | 6(r) | 12(r) | 42(s) | 35(s) | 13(i) | 6(r) | 35(s) | 38(s) |
| | M19 | 6(r) | 10(r) | 41(s) | 36(s) | 12(i) | 6(r) | 40(s) | 36(s) |
| | M40 | 6(r) | 8(r) | 40(s) | 35(s) | 14(i) | 6(r) | 24(s) | 38(s) |
| | M63 | 6(r) | 17(i) | 47(s) | 37(s) | 11(r) | 6(r) | 45(s) | 38(s) |
| | M66 | 6(r) | 12(r) | 42(s) | 38(s) | 12(i) | 8(r) | 39(s) | 38(s) |
| | M15 | 6(r) | 9(r) | 40(s) | 34(s) | 14(i) | 6(r) | 35(s) | 34(s) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| ***L. rhamnosus*** | M44 | 6(r) | 6(r) | 26(s) | 34(s) | 7(r) | 6(r) | 30(s) | 26(s) |
| ***L. rhamnosus GG*** | 9A | 6(r) | 6(r) | 34(s) | 10(r) | 8(r) | 6(r) | 30(s) | 29(s) |
| ***L. casei shirota*** | 11A | 6(r) | 6(r) | 28(s) | 41(s) | 9(r) | 6(r) | 37(s) | 25(s) |

[R]Resistance according to the EFSA's breakpoints (EFSA, 2008).

VAN: vancomycin (30 µg); CIP: ciprofloxacin (5 µg); Te: tetracycline (30 µg), Amp: ampicillin (10 µg), Cn: gentamycin (10 µg), K: kanamycin (30 µg), C: chloramphenicol (30 µg), E: erythromycin (15 µg); r: resistant; i, intermediate; s: susceptibility

**Table 6:** The number of strains of LAB species based on antibiotic susceptibility

| | | Number of isolates which are; | | | |
|---|---|---|---|---|---|
| Antibiotics | Species | Number of strains | Resistant | Intermediate | Susceptible |
| **VAN** | *L. plantarum* | 18 | 18 | 0 | 0 |
| | *L. paracasei* | 19 | 19 | 0 | 0 |
| | *L. rhamnosus* | 2 | 2 | 0 | 0 |
| | *L. casei shirota* | 1 | 1 | 0 | 0 |
| **CIP** | *L. plantarum* | 18 | 18 | 0 | 0 |
| | *L. paracasei* | 19 | 18 | 1 | 0 |
| | *L. rhamnosus* | 2 | 2 | 0 | 0 |
| | *L. casei Shirota* | 1 | 1 | 0 | 0 |
| **TET** | *L. plantarum* | 18 | 0 | 0 | 18 |
| | *L. paracasei* | 19 | 0 | 0 | 19 |
| | *L. rhamnosus* | 2 | 0 | 0 | 2 |
| | *L. casei Shirota* | 1 | 0 | 0 | 1 |
| **AMP** | *L. plantarum* | 18 | 0 | 0 | 18 |
| | *L. paracasei* | 19 | 0 | 0 | 19 |
| | *L. rhamnosus* | 2 | 1 | 0 | 1 |
| | *L. casei shirota* | 1 | 0 | 0 | 1 |
| **GEN** | *L. plantarum* | 18 | 17 | 1 | 0 |
| | *L. paracasei* | 19 | 4 | 15 | 0 |
| | *L. rhamnosus* | 2 | 2 | 0 | 0 |
| | *L.casei shirota* | 1 | 1 | 0 | 0 |
| **KAN** | *L. plantarum* | 18 | 18 | 0 | 0 |
| | *L. paracasei* | 19 | 19 | 0 | 0 |
| | *L. rhamnosus* | 2 | 2 | 0 | 0 |
| | *L. casei Shirota* | 1 | 1 | 0 | 0 |
| **CMP** | *L. plantarum* | 18 | 0 | 0 | 18 |
| | *L. paracasei* | 19 | 0 | 0 | 19 |
| | *L. rhamnosus* | 2 | 0 | 0 | 2 |
| | *L. casei shirota* | 1 | 0 | 0 | 1 |
| **ERY** | *L. plantarum* | 18 | 0 | 0 | 18 |
| | *L. paracasei* | 19 | 0 | 0 | 19 |
| | *L. rhamnosus* | 2 | 0 | 0 | 2 |
| | *L. casei shirota* | 1 | 0 | 0 | 1 |

Abbreviations: VAN: vancomycin (30 µg); CIP: ciprofloxacin (5 µg); TET: tetracycline (30 µg), AMP: ampicillin (10 µg), GEN: gentamycin (10 µg), KAN: kanamycin (30 µg), CMP: chloramphenicol (30 µg), ERY: erythromycin (15 µg)

### 3.6 Acidification in milk.

The acidification properties of all isolated LAB strains were investigated in pasteurized milk by pH measurement following 24 and 48 h fermentation (Table 8).

*L. paracasei* dropped pH to an average of 4.6 after 24 h and further to 3.9 after 48 h, while *L. plantarum,* and *L. rhamnosus* could achieve approximately an average pH of 5.2 and 4.7 after 24 and 48 h, respectively. Statistical analysis using one-way ANOVA showed a significant difference ($p<0.05$) in the pH reduction between the three species of LAB after 24 and 48 h fermentation period with p-value of $2.05 \times 10^{16}$ and $2.95 \times 10^{16}$ respectively.

**Table 7:** pH values of LAB strains at time 0 and after 24, 48h

| | | **pH (T0)** | **pH (T24)** | **pH (T48)** |
|---|---|---|---|---|
| ***L. plantarum*** | M95 | 6.64 | 5.26 | 4.85 |
| | M75 | 6.68 | 5.18 | 4.7 |
| | M87 | 6.68 | 5.15 | 4.72 |
| | M84 | 6.72 | 5.23 | 4.56 |
| | M96 | 6.73 | 5.41 | 4.7 |
| | M89 | 6.72 | 5.37 | 4.72 |
| | M88 | 6.68 | 5.13 | 4.61 |
| | M73 | 6.7 | 4.92 | 3.92 |
| | M94 | 6.69 | 5.18 | 4.59 |
| | M79 | 6.73 | 5.34 | 4.65 |
| | M93 | 6.74 | 5.07 | 4.75 |
| | M80 | 6.71 | 5.2 | 4.99 |
| | M86 | 6.71 | 5,15 | 4.78 |
| | M98 | 6.71 | 5.21 | 4.72 |
| | M22 | 6.72 | 5.41 | 4.93 |
| | M77 | 6.69 | 5.33 | 4.84 |
| | M92 | 6.68 | 5.41 | 4.81 |

| | | | | |
|---|---|---|---|---|
| | M85 | 6.69 | 5.12 | 4.77 |
| ***L. paracasei*** | M64 | 6.73 | 4.6 | 3.88 |
| | M35 | 6.72 | 4.62 | 3.88 |
| | M11 | 6.71 | 4.76 | 3.96 |
| | M36 | 6.71 | 4.61 | 3.9 |
| | M57 | 6.71 | 4.64 | 3.92 |
| | M70 | 6.71 | 4.58 | 3.91 |
| | M41 | 6.69 | 4.6 | 3.95 |
| | M65 | 6.72 | 4.78 | 3.91 |
| | M69 | 6.7 | 4.55 | 3.89 |
| | M38 | 6.75 | 4.77 | 3.91 |
| | M39 | 6.72 | 4.73 | 3.91 |
| | M47 | 6.7 | 4.56 | 3.92 |
| | M56 | 6.7 | 4.53 | 3.93 |
| | M59 | 6.7 | 4.59 | 3.91 |
| | M19 | 6.71 | 4.83 | 3.9 |
| | M40 | 6.71 | 4.71 | 3.94 |
| | M63 | 6.68 | 4.55 | 3.95 |
| | M66 | 6.69 | 4.53 | 3.91 |
| | M15 | 6.72 | 4.67 | 3.87 |
| ***L. rhamnosus*** | M44 | 6.68 | 5.11 | 4.46 |
| ***L. rhamnosus GG*** | 9A | 6.71 | 5.67 | 4.54 |
| ***L. casei shirota*** | 11A | 6.71 | 4.45 | 4.00 |
| **Control** | | 6.79 | 5.48 | 4.74 |

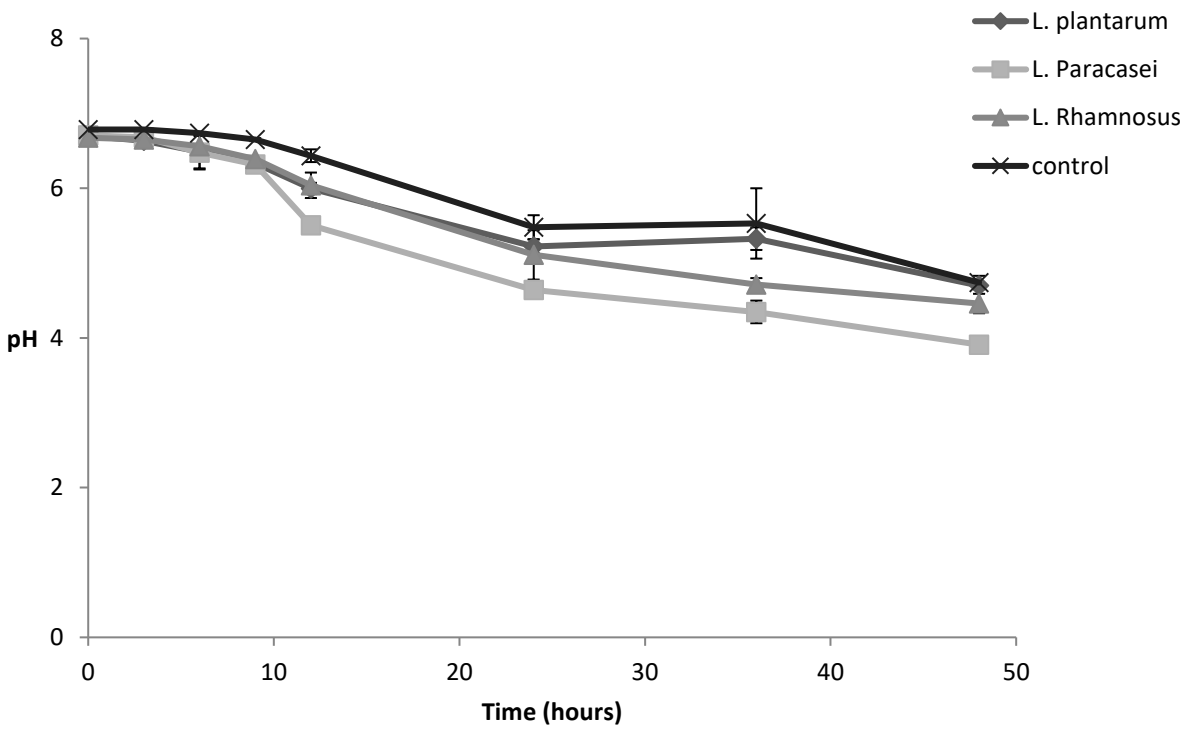



**Fig. 2: A**cidification of LAB strains cultured anaerobically in pasteurized milk at 37 ºC for 48 h. pH values were measured at 0, 24 and 48 h. Error bars in the graph represent the standard deviation of mean pH value of triplicate values for each strain. Where pasteurized milk without the inoculation of any bacteria was used as control.

### 3.7 Bile resistance

The 38 strains were grown for 24 h in sodium deoxycholate (bile salt). The *L. plantarum* and *L. rhamnosus* strains showed a minimum inhibitory concentration (MIC) of 0.6 % sodium deoxycholate while the *L. paracasei* and *L. casei shirota* strains showed a minimum inhibitory concentration (MIC) of 0.2 % sodium deoxycholate. The results were analysed according to (Chateau et al., 1994) who has proposed the separation of strains into 4 groups according to the delay in growth in the presence of bile acids which are: resistant strains (delay of growth $d \leq 15$ min), tolerant strains ($15 < d \leq 40$ min), weakly tolerant strains ($40 < d < 60$ min) and sensitive strains ($d \geq 60$ min). From the present study, we observed that 16 of the *L. plantarum* strains

were mildly tolerant to bile while two were sensitive. All strains of *L. paracasei* and *L. rhamnosus* were sensitive to bile (sodium deoxocholate).

**Table 8:** Minimum inhibitory concentration of isolates in sodium deoxocholate (Bile).

| **STRAINS** | | **MIC to Sodium deoxocholate (%)** |
|---|---|---|
| ***L. plantarum*** | M95 | 0.6 |
| | M75 | 0.6 |
| | M87 | 0.6 |
| | M84 | 0.6 |
| | M96 | 0.6 |
| | M89 | 0.6 |
| | M88 | 0.6 |
| | M73 | 0.6 |
| | M94 | 0.6 |
| | M79 | 0.6 |
| | M93 | 0.6 |
| | M80 | 0.6 |
| | M86 | 0.6 |
| | M98 | 0.6 |
| | M22 | 0.6 |
| | M77 | 0.6 |
| | M92 | 0.6 |
| | M85 | 0.6 |
| ***L. paracasei*** | M64 | 0.2 |
| | M35 | 0.2 |
| | M11 | 0.2 |
| | M36 | 0.2 |
| | M57 | 0.2 |
| | M70 | 0.2 |
| | M41 | 0.2 |
| | M65 | 0.2 |
| | M69 | 0.2 |
| | M38 | 0.2 |
| | M39 | 0.2 |
| | M47 | 0.2 |

| | | |
|---|---|---|
| | M56 | 0.2 |
| | M59 | 0.2 |
| | M19 | 0.2 |
| | M40 | 0.2 |
| | M63 | 0.2 |
| | M66 | 0.2 |
| | M15 | 0.2 |
| ***L. rhamnosus*** | M44 | 0.6 |
| ***L. rhamnosus GG*** | 9A | 0.6 |
| ***L. casei shirota*** | 11A | 0.2 |

**Table 9:** Classification of bacteria according to their resistance to bile (sodium deoxocholate)

| Group | I | II | III | IV |
|---|---|---|---|---|
| Delay of growth (d) | d≥ 15 min | 15<d< 40 min | 40<d<60 min | d≥60 min |
| ***L. plantarum*** | | | 16 | 2(M22&M98) |
| ***L. paracasei*** | | | | 19 |
| ***L. rhamnosus*** | | | | 2 |
| ***L. casei shirota*** | | | 1 | |

The strains were tested in triplicate for their resistance to sodium deoxycholate (bile). d denotes delay in growth between the control and culture with sodium deoxycholate; I, group of bile resistant strains; II, group of bile tolerant strains; III, group of strains with mild tolerance; IV, group of bile sensitive strains.

### 3.8 Biofilm formation

The strains were divided into two groups based on their ability to form biofilms (Fig. 3) of strong and weak biofilm formers based on their absorbance. Strains with $OD_{590nm} > 1$ were classed as strong producers whereas strains with $OD_{590nm} < 1$ are classed as weak producers. With respect to this classification, it can be inferred that of 38 strains, only 15 which were all *L. plantarum*, had a great capacity to create biofilms. On the other hand, 3 *L. plantarum* strains (M73, M93 and M80), were weak biofilm producers. Furthermore, all *L. paracasei* and *L. rhamnosus* strains showed weak biofilm production.

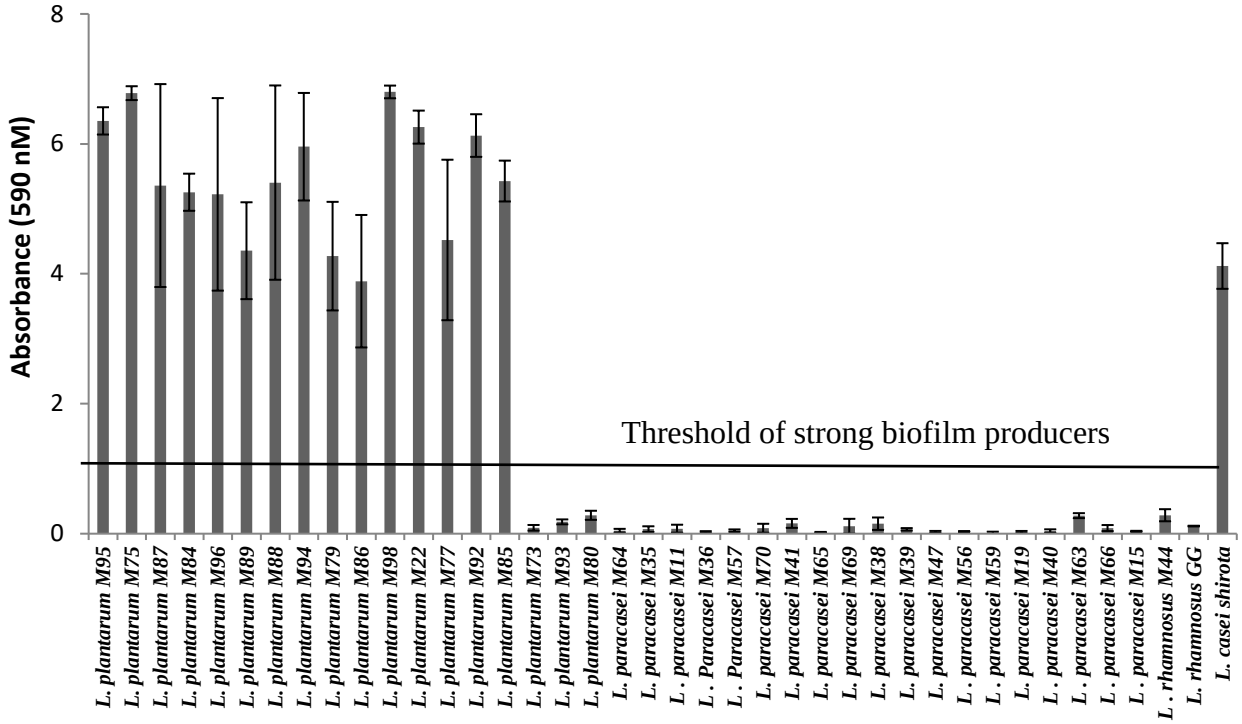



**Fig.3.** Biofilm formation of each LAB strain at 590 nm Absorbance. The strains were divided into strong and weak biofilm producers based on their absorbance value with $OD_{590} > 1$ represents strong biofilm producers and $OD_{590} < 1$ were weak biofilm producers. Error bars depict standard deviation of 3 biological and 3 technical replicates for each strain.

### 3.9 GABA production

The strains were tested for GABA production and they were divided into GABA producers (>1.5mM) and major GABA producers (> 2.5 mM). We observed that 16 out of the 18 (≃ 90 %) *L. plantarum* strains were major GABA producers while the *L. paracasei* and *L. rhamnosus* strains were non-GABA producers.

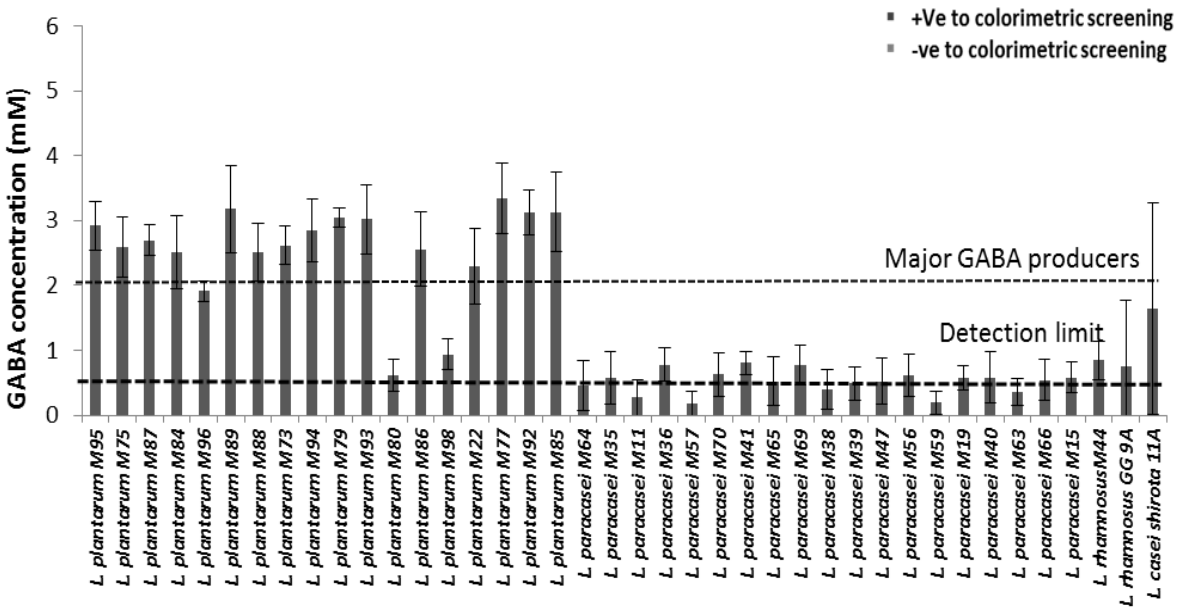
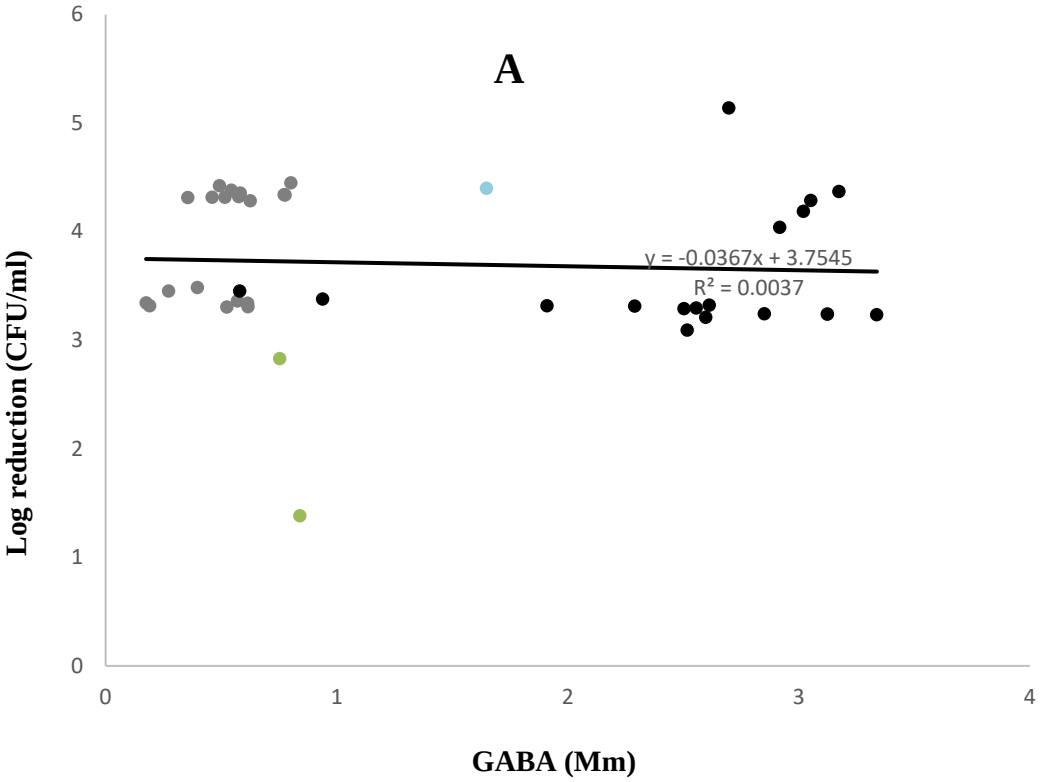
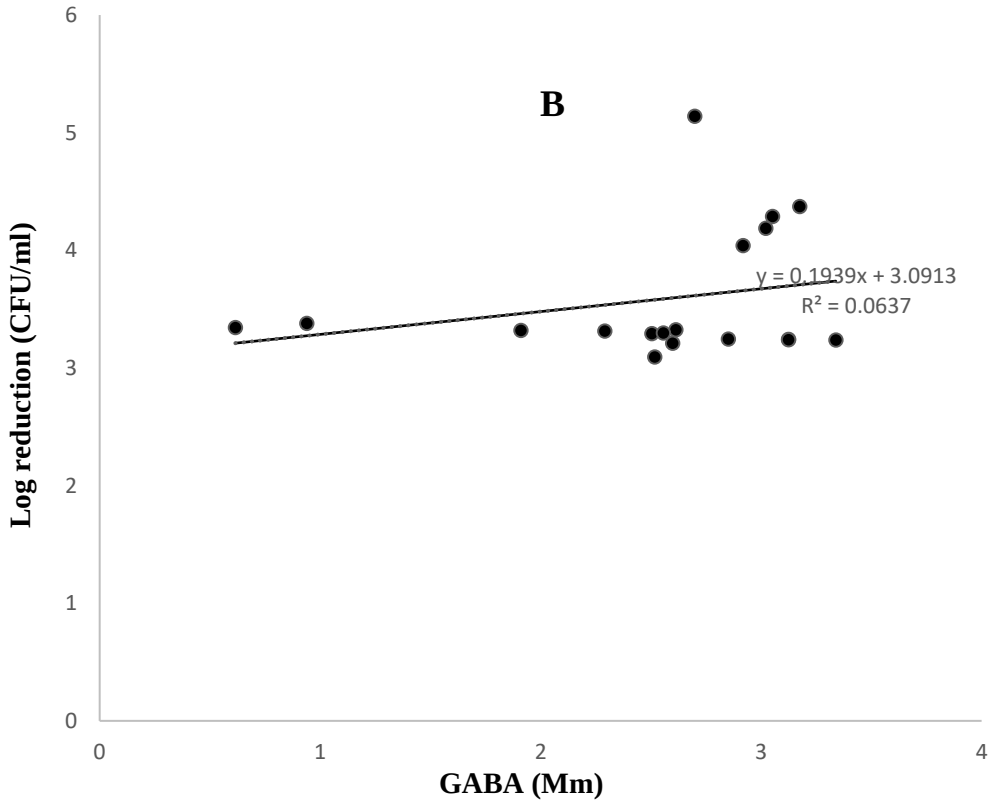



**Fig 4:** GABA production after 48 h for each strain in mM. Bars represent mean values of triplicate observations and error bars represent standard deviation of triplicate samples. Detection limit for GABA detection was 0.7 mM while major GABA producers were arbitrarily set those having a GABA concentration above 2.5 mM after 48 h of growth in MRS broth.

### 3.9.1 Correlation between GABA production and Log reduction when isolates were challenged at pH 2.5 for 1 h with 1M HCl

The mechanism used for resistance by microorganism under acidic stress is the glutamate decarboxylase. Therefore, as part of the probiotic property we correlated the GABA production to the acid resistance (i.e log reduction) of each strain and we found that there was no correlation between GABA production and log reduction although there was a distinct separation of the strains with the plot.


A
Log reduction (CFU/ml)
GABA (Mm)
y = -0.0367x + 3.7545
R² = 0.0037
0
1
2
3
4
5
6


B
Log reduction (CFU/ml)
GABA (Mm)
y = 0.1939x + 3.0913
R² = 0.0637
0
1
2
3
4
5
6

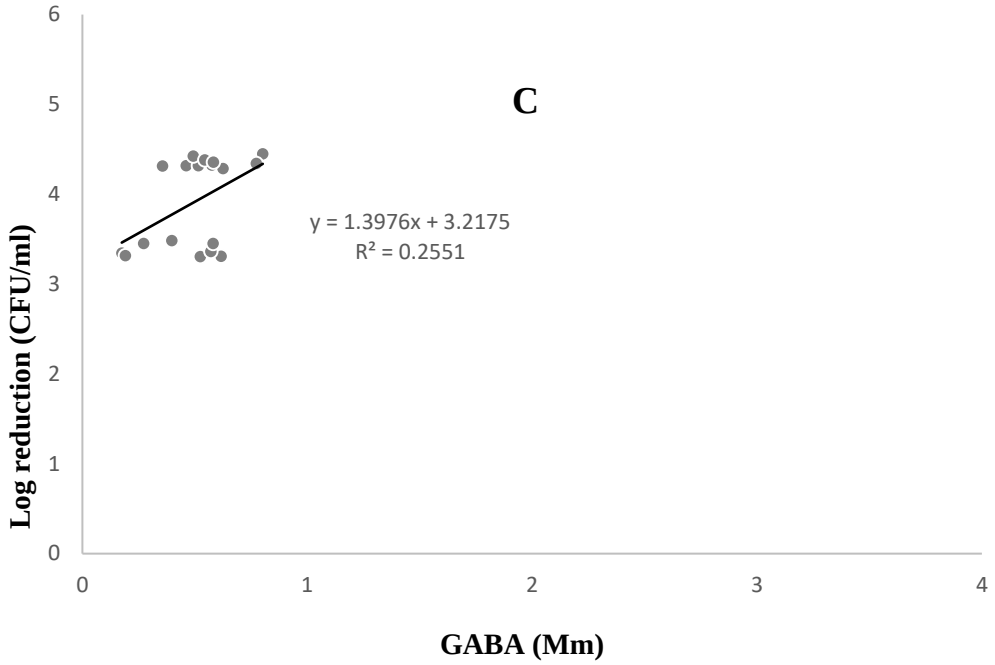



**Fig 5:** Correlation between GABA (Mm) concentration and Log reduction (cfu/ml). (A) the correlation with all the strains where L.plantarum is in black, L. paracasei in grey, *L.casei chirota* in blue and *L. rhamnosus* in green. (B) Correlation with *L.plantarum* and (C) correlation with *L.paracasei*.

### 3.9.2 Batch culture fermentation with two (*L. plantarum* M77 & M84) major GABA producers (> 2.5 mM).

Batch culture fermentation was used to investigate if strong GABA producer LAB strains could enhance GABA levels in the human colon, following their delivery. Therefore, we selected two major GABA producers (*L. plantarum* M77 & M84) and used as cultures for batch culture fermentation to determine if there will be an increase in GABA production compared to the control. The results showed that there were higher levels in GABA production with both strains but only at 48 h and lower levels at 24 h (Fig. 6). However, these differences in GABA levels compared to the control were not statistically significant ($P > 0.05$). The above might suggest that these strains, although result in a slower increase in GABA levels which however, reach the highest value at 48 h and not at 12 h as occurs in the control. Furthermore, we did not observe a reduction in GABA levels as we saw in the control.

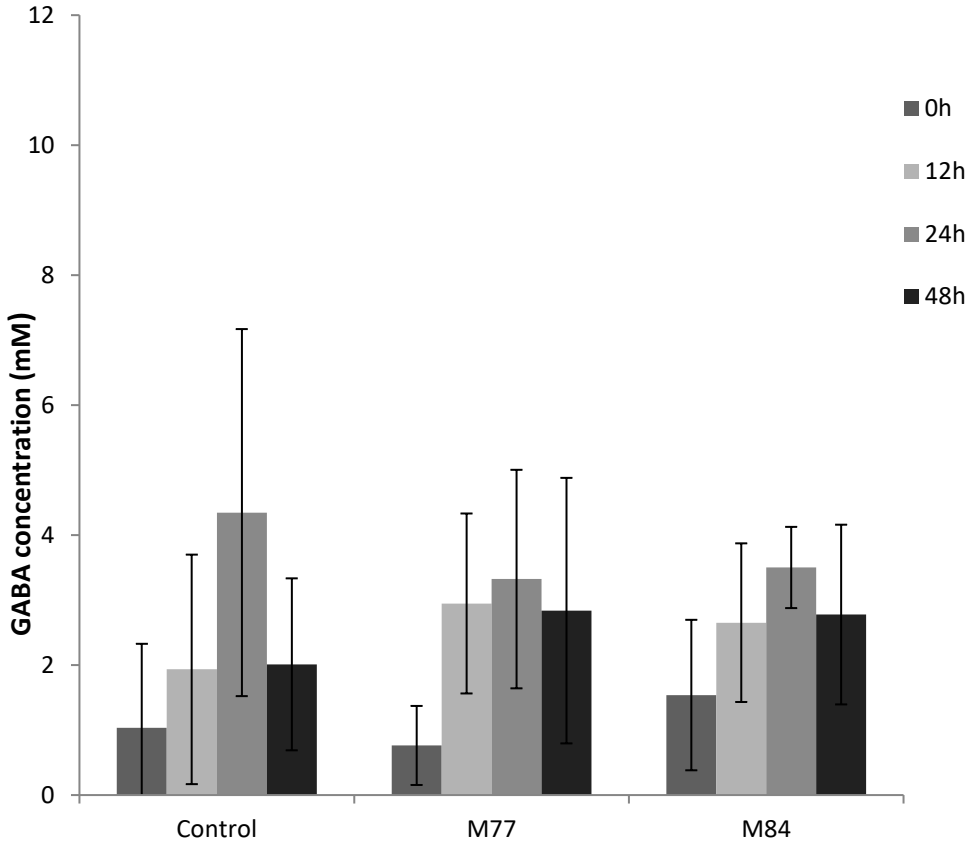
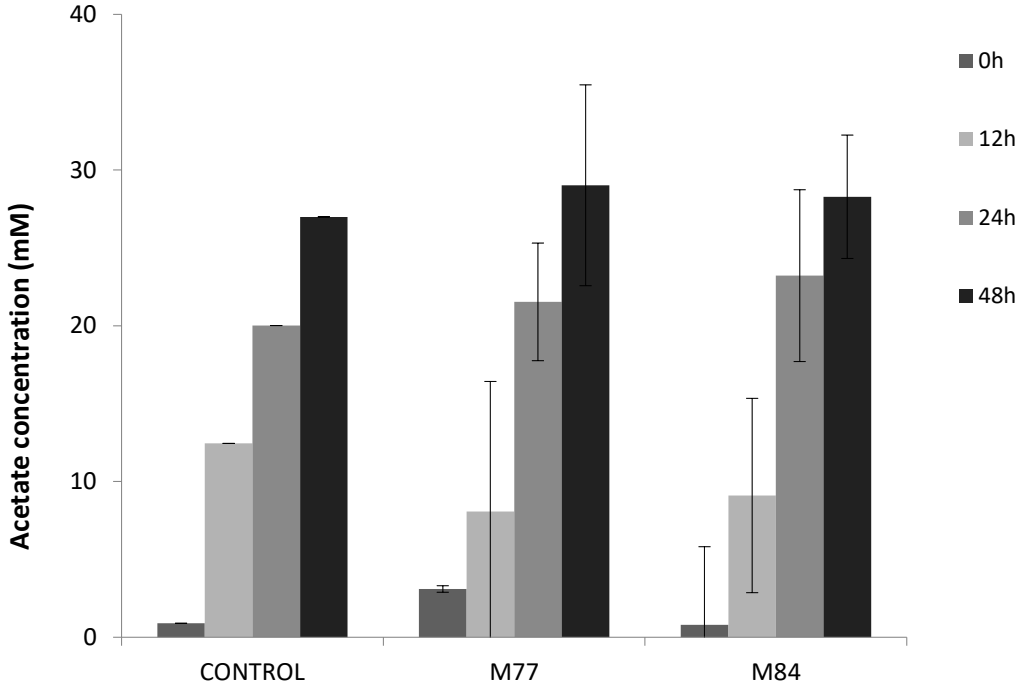



**Fig 6:** Bars represent average GABA levels after batch culture fermentation with *L. plantarum* (M77 & M84). Error bars represent standard deviation between five donors. T-test showed no significant difference between the strains and control at each time point as in all cases P >0.05.

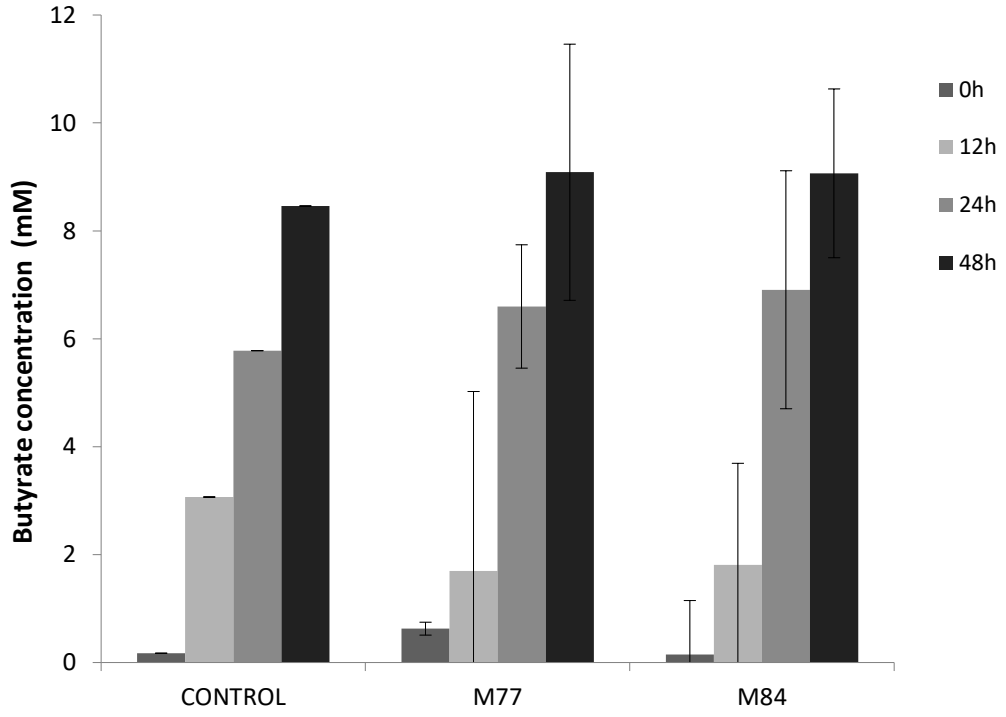
### 3.10 Short chain fatty acid production

The SCFA production was done to determine if there was an increase in acetate, butyrate and propionate production during the 48 h batch culture fermentation with the two major GABA producing strains (*L. plantarum* M77 & M84). The results (Fig. 7) showed there was no significant difference in the production of acetate, butyrate and propionate between the two strains and the control at each time point using T-test. However, we can see a decrease in the production of each compound at 12 h and a slight increase in the compounds at 48 h which however were not accompanied by statistical significance.

A


40
30
20
10
0
Acetate concentration (mM)
CONTROL
M77
M84
0h
12h
24h
48h

B


12
10
8
6
4
2
0
Butyrate concentration (mM)
CONTROL
M77
M84
0h
12h
24h
48h

C

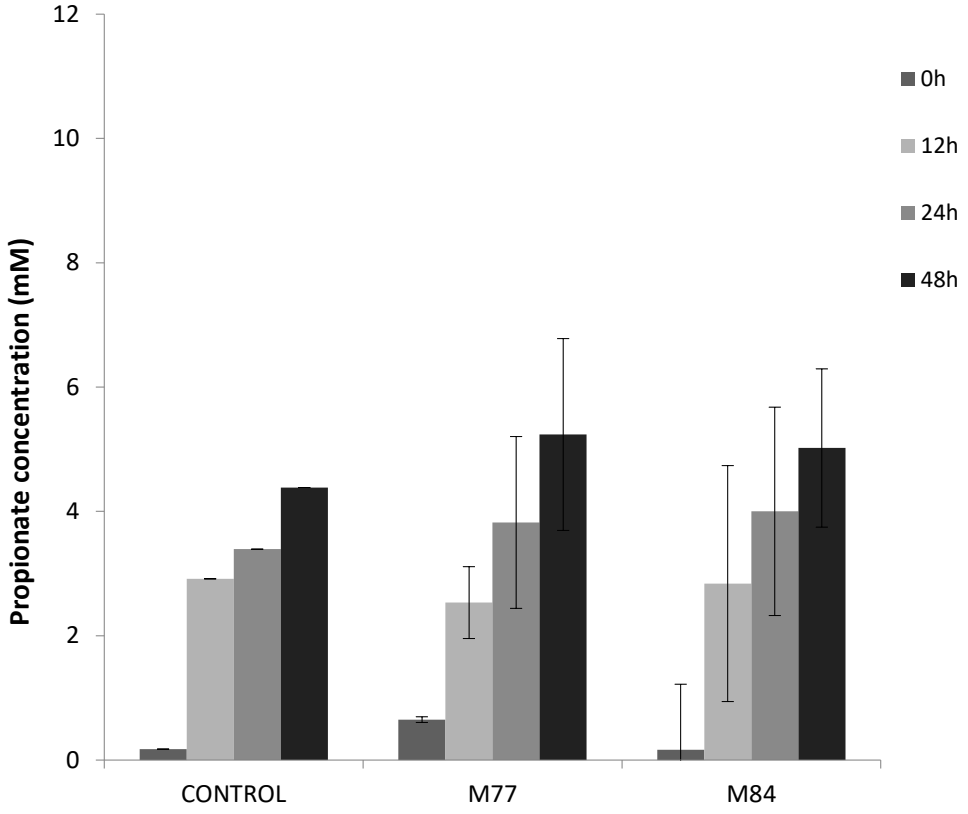



**Fig 7:** Short chain fatty acids (SCFAs) concentration (mM) after batch culture fermentation with *L. plantarum* (M77 & M84). Bars represent mean concentration of acetate (A), butyrate (B) and propionate (C) while error bars represent standard deviation between five donors. T-test showed no significant difference between the SCFA levels in the presence of strains and control at each time point ($P < 0.05$).

# 4. Discussion

In this study, 38 strains of Lactic acid bacteria isolated from the human gut microflora and two reference strains used as control were tested for their probiotic properties. The isolated strains were identified using the Biomerieux API 50CH identification kit (Table 2) and biochemical profile of faecal isolates on the *apiweb*$^{TM}$ identification software was also performed to be able to identify the strains based on the sugars they metabolise (Table 1) and from these we were able to select 18 *L. plantarum*, 19 *L. paracasei* and 1 *L. rhamnosus* strains for the purpose of this study. The tests were performed to determine their survival in simulated gastric conditions. The strains were tested for GABA production which is an inhibitory neurotransmitter that has been widely studied for its various health benefits (Hayakawa et al., 2004, INOUE et al., 2003, Tanaka et al., 2009). A look into all the tests done including GABA production can open up the potential for these strains to be used as either probiotics or starter cultures in developing functional or fermented foods (Kim and Kim, 2012). Since the health benefits of GABA have been explored, this has led to an increase in the production of GABA enriched by the food processing industry. Therefore, it is paramount to study and isolate LAB strains that are GABA producers and explore their applications to a greater extent in fermented foods (Tung et al., 2011, Osborn).

To be classed as a probiotic, an organism must be tested for its safety for human consumption and must remain viable while passing through the intestinal tract and it should have a population of $10^6$-$10^7$ cfu/g when reaching the gut. These numbers would have ability to deliver the required health benefits to the host (Pavli et al., 2016). Bearing this in mind, one of the major problems bacteria face during survival in the gut is the acidic conditions in stomach. Furthermore, the inhibitory effects of bile in the duodenum pose an additional problem (Argyri et al., 2013). From recent studies, the acceptable limit for bile concentration ranges from 0.15% to 0.5% as this is the range in which the physiological conditions are met in the gastrointestinal

tract (GIT) (Vizoso Pinto et al., 2006). From our results (Table 9), we found that the *L. paracasei* strains were more sensitive to sodium deoxocholate (bile salt) with MIC of 0.2 % while the *L. plantarum* strains are mildly tolerant to bile salts with MIC above the limit (0.5 %). Comparing the bile tolerance with acid resistance (Fig. 1) we found that *L. plantarum* and *L. rhamnosus* strains that were mildly tolerant to bile salts were also resistant to acid at pH 2.5 with 3 and 1 log reduction of CFU/ml respectively at 60 min while the *L. paracasei* strains showed sensitivity to both bile and acid (4 log reduction). Various studies have reported the ability of Lactobacilli to be able to survive at a pH of 2.0 to 3.0, which mimics the physiological conditions in the stomach. The sensitivity to acid and bile salts of each Lactobacilli are therefore strain-dependant (Belicova et al., 2013)

According to (Korhonen, 2010), there are several methods of assessing the antimicrobial susceptibility of bacterial strains but due to cost, effective and reliable properties as well as rapid results we decided to use the disc diffusion method and from our results (Table 6) all LAB strains were determined as resistant to vancomycin (30 µg) and kanamycin (30 µg) while they exhibited sensitivity to tetracycline (30 µg), ampicillin (10 µg) and erythromycin (15 µg). *Lactobacillus* species have high level of natural resistance to vancomycin as a result of gene clusters that located on a plasmids which are transferable (Klein et al., 2000). For safety purposes, bacteria's resistance to specific antibiotics must not be transferable and according to the technical guidance from EFSA, intrinsic resistance and resistance due to mutation of chromosomal genes confers low risk to public health and such probiotic strains should be accepted for use in food processes (EFSA, 2008). Although in the present study *Lactobacillus* species showed resistance to vancomycin, ciprofloxacin and kanamycin, this resistance is intrinsic resistance as also seen by the reference strains (Sharma et al., 2017, Mathur and Singh, 2005).

We also investigated the biofilm formation ability of the LAB strains because with biofilm production, bacteria could colonise or attach to a surface that could be a mucosal membrane in the gastrointestinal tract which is a desirable characteristic when considering a bacteria to be used as probiotic also, the formation of biofilm from LAB strains could also disrupt or curb the colonisation by pathogenic bacteria through mechanisms such as immunomodulation or through their ability to competitively exclude the pathogenic bacteria from the surfaces (Macfarlane and Dillon, 2007, Salas-Jara et al., 2016). The results (Fig. 3) showed that *L. plantarum* is a strong biofilm producer. Although, (Zhang et al., 2013) reported a significant correlation between biofilm forming ability of LAB strains and antibiotic resistance to tetracycline, erythromycin, ampicillin and streptomycin. However, we found that none of LAB strains was resistant to tetracycline, erythromycin and ampicillin though 15 out of 38 were able to produce high amount of biofilm. As seen in Fig 3. 15 out of 18 strains of *L. plantarum* exhibited strong biofilm formation whilst *L. paracasei* and *L. rhamnosus* strains were included in the weak producer group. Therefore, from this present study we could not find any relationship between antibiotic resistance and biofilm formation. Moreover, Kubota et al. indicated that biofilm formed by *L. plantarum* subsp. *plantarum* JCM1149, exhibited resistance up to 11 % and 40 % in the presence of acetic acid and ethanol, respectively (Kubota et al., 2008). It can be inferred from the study that biofilm may be thought as a defence or protective mechanism which could possibly have been developed under stress condition such as acidic conditions and high temperature.

Another probiotic property we focused on was their antimicrobial activity as it has been reported that bacteria produce antimicrobial compounds such as bacteriocins, hydrogen peroxide, organic acids and ethanol (Kralik et al., 2018, Pisano et al., 2014). In general, diffusion methods, dilution methods and thin layer chromatography (TLC), time-kill test and flow cytofluorometric method are widely used methods of detecting antimicrobial activity. For

the purpose of this study, the disc diffusion method was used and *L. monocytogenes* was used as a model organism to test the antimicrobial properties of the strains. Therefore, the results showed that *L. rhamnosus* exhibited no inhibition whereas *L. plantarum* and *L. paracasei* exhibited a small inhibition zone diameter. Nonetheless, statistically there was no significant difference between species. In this study, *L. paracasei* and *L. rhamnosus* did not show inhibition against *L. monocytogenes*. In contrast, (Abbasiliasi et al., 2009) reported that *L. paracasei*, *L. plantarum* and *L. rhamnosus* showed very strong inhibition against *L. monocytogenes*, respectively. Antilisterial activity of LABs differs depending on the metabolites that are being produced by each species or strain. It has been reported that active peptides such as nisin (class I) and pediocin-like (class IIa) are inhibitors of *L. monocytogenes* although, it has been observed that in laboratory media some strains of *L. monocytogenes* have developed a tolerance to nisin and pediocin like substances (Abbasiliasi et al., 2009, Parente and Ricciardi, 1999, SLOŽILOVÁ et al., 2014). Therefore, there could be a possibility that the LAB used are not antimicrobial-active strains or may be the *L. monocytogenes* has developed a tolerance to the peptides produced.

The acidification property of LAB in milk was also investigated since it is an essential characteristic for starter cultures used during fermentation processes. It has been suggested by (Sodini et al., 2002) that fermentation time of milk should be determined when the milk attains a pH of 4.55. Therefore, from our results (fig 2), the fermentation time for *L. paracasei* is 24 h, and above 48 hours for both *L. plantarum* and *L. rhamnosus as the* acidifying behaviour of species throughout 48 h incubation period was shown (Fig. 2). As seen, *L. paracasei* showed the highest acidification capability with an average pH of 4.6 and 3.9 after 24 and 48 h whereas *L. plantarum* and *L. rhamnosus* reached an average pH of 4.7 after 48 h, respectively. Sodini et al. also reported a fermentation time of 23.5 h for *L. rhamnosus* LR35 in milk samples. This acidifying capability was lower than *Streptococcus thermophilus* ST7, *Lactobacillus*

*bulgaricus* LB12, *Lactobacillus acidophilus* LA5 which had a fermentation time of 11 h. It was also reported that the strains that had higher fermentation time in milk were more stable during storage that the strains with lower fermentation time therefore more work needs to be done on the shelf-life of the milk and stability of the strains during storage. It should also be taken into consideration that fermentation process is provided via a mixture of culture and each of them has different purpose such as aroma enhancement or texture improvement. Therefore, further studies need to be done to determine their contributions to aroma profile and texture of products in different fermented processes.

We also decided to look at the γ-aminobutyric acid (GABA) production of these strains because of the health benefits of this amino acid. As such these strains could possibly be used to develop fermented functional foods. We found that *L. plantarum* strains were major GABA producers compared to *L. paracasei* and *L. rhamnosus* (Fig. 4). Various studies have been able to prove the production of GABA from different *Lactobacillus* strains isolated from various fermented foods such as cheeses, kimchi etc. Although, there was no GABA production in *L. paracasei* isolated from the gut as the production of GABA from gut microbiota has always been generally classified under the Lactic acid bacteria group which was suggested that Lactobacilli are included in the highest GABA producing strain from the gut but the metabolic activities are more likely to be strain specific rather that genus related (Li and Cao, 2010). The fact that there was no GABA production by *L. paracasei* could possibly be as a result of culturing in 10 mM of glutamate as studies have shown high production of GABA in *L. paracasei* of up to 302 mM using 500 mM of monosodium glutamate (Wu et al., 2018, Kook and Cho, 2013). Furthermore, few strains isolated from the human gut microbiota have been reported to have the ability to produce GABA and likewise suggested that the genes encoding GAD could possibly be present in significant proportion of the human gut microbiota (Mazzoli and Pessione, 2016). Comparing the properties of the LAB strains with the reference strains especially *L. casei*

*Shirota* which is a widely used probiotic. The strains especially the *L. plantarum* showed similar characteristics in probiotic properties such as acid resistance, acidification and biofilm formation with the *L. casei Shirota* strain.

From all these results, we decided to select two of the GABA producing strains carry out a batch culture fermentation to see if they could influence the levels of GABA in an environment simulating the gut. We also looked in parallel at the possible effects of these strains on the levels of short chain fatty acids (SCFAs) such as acetate, butyrate and propionate production. These major SCFAs result from the fermentation of both carbohydrates and amino acids in the human gut. However, from our results (Fig 5) we found a slight increase in GABA production at 48 h although the increase in both GABA and SCFA production were not statistically significant. Therefore, we suggest that more work need to be done to confirm if this slight increase was from the *L. plantarum* strain used for fermentation. Likewise, there was no increase in SCFA (Fig 6A, 6B & 6C) which could be as a result of the media used for fermentation. As basal medium is considered a basic medium and does not have enough nutrients to support the production of SCFA. Hence, the reason for not seeing an effect on SCFA in the batch culture fermentation.

Finally, we sent the 2 selected *L. plantarum* strains for whole genome sequencing (table 3) to back up our results from the identification done using the Biomerieux API 50CH kit (table 2) and also to determine the genes responsible for GABA production and also to see if the genes responsible for resistance to specific antibiotics are intrinsic and also see if there will be a link with the chromosomal genes and the various tests carried out using these two selected strains.

# 5. Conclusion

In conclusion, it is indicated that LABs isolated from human gut might be considered as good probiotic candidates due to their strong resistance to low pH and bile salt, 16 out of 38 LAB strains, which are the *L. plantarum*, showed high GABA producting ability which were also classed as strong biofilm producers and were more resistant to acid. On the other hand, *L. paracesi* showed higher acidification compared to *L. plantarum* and *L. rhamnosus*. As a result, every LAB strain might be used as probiotic for different purposes. Therefore, more in vivo and molecular work needs to be carried out to investigate these LAB strains to be able to fully understand and clarify their beneficial properties as well as their safety as several LAB strains are used as starter culture and also for probiotics, and they have been known to produce GABA during metabolism but they also produce some biogenic amine compounds such as histamine, tyramine and putrescine, which are toxic to the human body (Wu et al., 2018). Additionally, more work is needed to assess their technological features in food process in order to develop value added products such as fermented foods and beverages or probiotic / functional products.

# 6. Acknowledgment

The authors would like to thank all colleagues at the Microbiology research team, Food and Nutritional Sciences, University of Reading. Society for applied microbiology (SFAM) for the studentship grant give and MicrobesNG for the genome sequencing provided *(http://www.microbesng.uk).* Also, Nur Yesilyurt who conducted most of the *in-vitro* work as part of her MSc research project.

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# Chapter 4

# Modulation of the gut microflora γ-aminobutyric (GABA) and short chain fatty acids (SCFAs) production through feed in pH-controlled single stage continuous culture fermentation and static batch culture fermentation

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## Abstract

Gamma aminobutyric acid (GABA) which is the major inhibitory neurotransmitter in the central nervous system and has been widely studied due to its various health benefits such as lowering blood pressure, reducing anxiety, depression and many others. However, research mainly focuses on orally consumed GABA, but recently it has been shown that members of the gut microbiota produce high levels of GABA which could affect the host and play a role in the gut-brain axis.

In previous experiments conducted by colleagues, we have identified in an individual bacterium (*Listeria monocytogenes*) that amino acids might affect the output of the glutamate decarboxylase (GAD) system. If this is a general behaviour of the gut microbiota, we might be able to manipulate GABA production in the gut. Therefore, we assessed GABA and short chain fatty acid (SCFA) production by the gut microbiota using batch culture fermentation in basal (pH-controlled) and brain heart infusion (non-pH-controlled) medium following supplementation with amino acid sources such as casamino acids, tryptone, peptone and yeast extract.

Hence, we found that in basal medium, supplementation with all compounds except yeast extract increased the GABA production, with casamino acids yielding the highest levels of GABA with about 8-fold increase in concentration at 48 h. Also, we found that the addition of casaminoacids, tryptone, peptone yeast extract and L-cysteine increased the production of acetate, propionate and butyrate after 48 hrs unlike L-tryptophan while in the non-pH-controlled fermentation, the addition of these amino acids increased GABA production with casamino acids being the most effective with an increase of approximately 3–fold and 6-fold at 12 h and 24 h respectively. Consequently, the main SCFAs (acetate, propionate and butyrate)

also increased with the amino acids increasing the concentration of acetate, butyrate and propionate by at least 2-fold by 48 hr.

In Conclusion, GABA and SCFAs are metabolites produced during bacterial fermentation in the gut. Modulating the production of these metabolites in the gut can possibly be a link between the diet, gut microbiota and human physiology.

# 1. Introduction

The gut microbiota comprises trillions of bacteria present in the gastrointestinal tract. These bacteria have been shown to play a significant role in regulating the immune system, influencing brain development as well as the function of the central nervous system of the host through the gut-brain axis (Strandwitz, 2018, Fung et al., 2017). In addition, studies, which have majorly been carried out in animal models, have given evidence of the role of the gut microbiota in influencing the development of diseases of the central nervous system through the gut brain axis (Fung et al., 2017, Evrensel and Ceylan, 2015).

Gut microorganisms are able to produce and deliver neuroactive substances such as γ-aminobutyric acid (GABA) which could possibly affect elements of the gut brain axis that is the link between the gastrointestinal tract (GIT) and central nervous system (CNS). These metabolites, as well as other possible stimuli that might affect the vagus nerve seem to be the link between the gut and brain (Mayer et al., Holzer and Farzi, 2014). However, it is not known the actual mechanism by which this takes place. However, it is well-known that the gut microbiota is able to modulate the gut brain axis through the production of neurotransmitters and neuromodulators and thus, studies have identified important microbial genera that produce bioactive compounds. For example *Lactobacillus* and *Bifidobacterium* genera have been reported to produce GABA while *Escherichia, Bacillus* and *Saccharomyces* produce dopamine and/or noradrenaline, *Candida, Streptococcus, Escherichia* , *Enterococcus*, *Candida*, and *Streptococcus* produce serotonin, *Bacillus and Serratia* produce dopamine and *Lactobacillus* also produces acetylcholine (Barrett et al., 2012, Park et al., 2013, Holzer and Farzi, 2014, Cerdó et al., 2017). One of the most interesting compounds produced by the gut microbiota and could have positive health effects to humans is GABA.

GABA is an inhibitory neurotransmitter, thereby functioning effectively by providing a relaxing effect on humans and other mammals by inhibiting the nerve cells in the brain from receiving a stimulatory effect, which in turns causes the patients to maintain a relatively calm and relaxed state (Harris and Allan, 1985). GABA as an inhibitory neurotransmitter regulates a significant number of the brain functions, therefore a disruption in the GABAergic inhibition could potentially be implicated in various diseases (Ting Wong et al., 2003). The production of GABA has been studied for its physiological and psychological functions in both human and animal models. One of its most interesting properties is its ability to regulate blood pressure in both hypertensive rats and human intervention trials (Hayakawa et al., 2004, Inoue et al., 2003, Tanaka et al., 2009). Also, a perturbation in GABA production has been linked with the onset of various diseases such as epilepsy, Parkinson's disease and Alzheimer's disease (Ting Wong et al., 2003, Battaglioli et al., 2003). Furthermore, GABA has been shown to prevent sleeping disorders, mood disorders, and depression (Ting Wong et al., 2003, Sasaki et al., 2006, Krystal et al., 2002, Bjork et al., 2001). In addition, GABA has been shown to slow down the proliferation of cancer cells, and it is also considered to help in suppressing tumours (Schuller et al., 2008, Oh and Oh, 2004, Kleinrok et al., 1998) .

While GABA plays an important role in the nervous system of mammals, in bacteria GABA plays a completely different role. A variety of bacteria utilise the glutamate decarboxylase (GAD) system which is one of the most important acid resistance systems and GABA is its by-product. This system increases the intracellular pH and helps the cells survive at acidic conditions (Fung et al., 2017, Feehily and Karatzas, 2013). Studies on the human microbiome have shown that the GAD system is present in a considerable proportion of members of the human gut microbiota (Mazzoli and Pessione, 2016). The GAD system catalyses the irreversible decarboxylation of glutamate to GABA. During the decarboxylation process, protons are consumed leading to an increase of the intracellular pH. As such, the GAD system

plays an important role in survival in acidic environment of the stomach through which, the gut microbiota have to pass in order to establish themselves in lower parts of the intestinal tract (Pokusaeva et al., 2017, Mazzoli and Pessione, 2016, Karatzas et al., 2012).

It is widely understood that the kind of diet and the supply of nutrients affects significantly the make-up of the gut microbiota. As such, this can have a direct effect on the health of the host. (Huang et al., 2016, Etxeberria et al., 2013). However, this is extremely complex to understand as it is a system of human cells that utilise the nutrients provided by the diet in addition to the metabolites produced by gut microbiota. It has been reported that subjects consuming protein-rich and high saturated fat diets have an increased level of *Bacteroides* genera (Yatsunenko et al., 2012). Furthermore, the level of *Prevotella* is known to increase in children ingesting high-fibre diet (De Filippo et al., 2010). Preliminary studies suggest that manipulation of the gut microbiota via various dietary interventions can alter the function and composition of the gut microbiota and this may in turn impact the levels of metabolites such as GABA produced by the gut microbiota (David et al., 2014). Also, environmental factors or host functions and dietary foods ingested can influence the make-up and the overall metabolism of the gut microbiome. Therefore, the interactions between the host, intestinal microflora and food ingested should be considered when modulating the functions and metabolism of the gut microbiota. These environmental factors are crucial in maintaining homeostasis in the ecosystem, therefore more work needs to be done in investigating and giving a better understanding on how specific compounds modulate or influence the gut microbiome (Bernalier-Donadille, 2010, David et al., 2014).

Since GABA is one of the molecules with major positive health effects which have been proven in human and animal subjects as well as in cancer cells in laboratory levels various studies have focused on increasing its levels in fermented products (Lim et al., 2017, Siragusa et al., 2007b) and which is the mechanism that this ingested GABA can elicit these positive health effects.

Another line of research looks at the production of GABA by the gut microbiota, by looking mainly at the main contributors. However, there is very little research on how the gut microbiota and these main contributors could be modulated to produce higher levels of GABA or other compounds such as short chain fatty acids (SCFAs) in the gut and this is what we investigate in the present work. In that way, the gut microbiota could be seen as an internal organ that is able to produce GABA among other metabolites that could have positive health effects. Such work could result in interventions that could help this organ elicit positive health effects to the host, apart from the supplementation of the compounds through diet.

# 2. Materials and methods

### 2.1 pH Controlled Single Stage culture fermentation

The basal nutrient medium was supplemented with 13 g/l of casamino acids or tryptone, or peptone or yeast extract or 10 g/l of L-cysteine or L-trypthophan. The latter medium contained 2 g/l peptone water, 2 g/l yeast extract (Oxoid, Hampshire, UK), 0.1 g/lNaCl, 0.04 g/l $K_2HPO_4$, 0.04 g/l $KH_2PO_4$, 0.01 g/l $MgSO_4.7H_2O$ (Fischer Scientific, Loughborough, UK), 0.01 g/l $CaCl_2.6H_2O$, 2 g/l $NaHCO_3$ (Fischer scientific, Loughborough, UK), 0.05 g/l hemin (dissolved in a few drops of 1 mol $l^{-1}$ NaOH), 0.5 g/l cysteine.HCl, 0.5 g/l bile salts , 2 g/l Tween 80, 4 ml resazurin (0.025g/100ml, pH7) and 10 μl vitamin $K_1$ and then autoclaved. Basal nutrient medium was prepared with chemicals obtained from Sigma-Aldrich, (Poole, UK) unless otherwise stated.

#### 2.1.1 Fermentation

Sterile batch culture fermenters (100 ml) were aseptically filled with 45 ml of the prepared medium and oxygen free $N_2$ gas was pumped into the media which were continuously stirred overnight to create anaerobic conditions. Ten percent (w/v) of faecal slurry was prepared by taking fresh faecal samples from 5 healthy volunteers (without antibiotics for 6 months) each sample was diluted with phosphate saline buffer (pH 7.4) and mixed in a stomacher (Seward,

stomacher 80, Biomaster) for 2 min at normal speed. Five ml of the faecal slurry was then inoculated into 45 ml basal medium in the batch culture fermenters at 37 $^{o}$C and pH was maintained between 6.7 - 6.9 representing the distal colon. One ml samples were taken at 0, 12, 24, & 48 h for GABAse analysis, SCFA analysis and 16S gene amplification and sequencing.

**2.2 Non- pH controlled Static batch culture fermentation**

One litre of brain heart infusion (BHI) broth (Lab M, Lancanshire, UK) was supplemented with 13 g casamino acids (Fisher Scientific, Loughborough, UK), or tryptone, or peptone, or yeast extract, or 10 g of L-cysteine or a similar amount of L-trypthophan and then autoclaved. All compounds used were obtained from Sigma-Aldrich, Poole, UK unless otherwise stated.

The autoclaved media was placed in the anaerobic cabinet over night at 37 $^{o}$C to make it anaerobic before inoculation. Ten percent (w/v) of faecal slurry was prepared by taking fresh faecal samples from 5 healthy volunteers (not taken antibiotics for the last 6 months). Each faecal sample mixed in the stomacher (Seward, stomacher 80, Biomaster, West sussex, UK) for 2 min at normal speed in phosphate saline buffer (pH 7.4). One ml of the faecal slurry was then inoculated in 9 ml BHI in sterile Hungate tubes and 1 ml each of samples was taken at 0, 12, 24, & 48 h for GABAse analysis, SCFA analysis, 16S gene amplification and sequencing

**2.3 GABA analysis**

GABA analysis was performed according to (Tsukatani et al., 2005) and (Karatzas et al., 2010). The 1 ml culture sample that was taken from the fermentation vessels, it was centrifuged at 11337×g for 10 min. The supernatants were stored in Eppendorf tubes and kept at – -80 $^{o}$C until they were required for analysis. GABAse master mix was prepared as such to contain 80 mM Tris (base) amino methane, 2 mM α-ketoglutarate,750 mM sodium sulphate, 10 mM dithiothreitol (DTT), 1.4 mM β-nicotinamide adenine dinucleotide phosphate (NADP) and 0.3 g/l of the enzyme GABAse from *Pseudomonas fluorescens* (Sigma Aldrich,

UK) in sterile water. Ninety µl of the master mix was placed in each one of the wells of a 96-well plates and subsequently, 10 µl of the defrosted supernatant was added. In parallel, GABA standard solutions containing known concentrations of GABA (0, 2, 4, 6, 8 and 10 mM) were prepared, added in wells containing the master mix and run alongside the samples. The plate was then placed in a Sunrise plate reader (Sunrise, TECAN, Mannedorf, Switzerland) set at 37 $^{o}$C where absorbance at 340 nm was monitored every 2 min for 3 h and data was recorded using Magellan software (TECAN, Mannedorf, Switzerland) and analysed using Microsoft Excel.

**2.4 Short chain fatty acid (SCFA) analysis**

The SCFA in faecal samples was measured using Gas Chromatography- Mass Spectrometry (GC-MS) detection and the derivatisation method was modified from (Richardson et al., 1989).

Fifty µl of 0.1 M 2-ethylbutyric acid solution was added to 1 ml of faecal sample in Hungate tubes and vortexed. The mixture was acidified with 0.5 ml concentrated HCl (38 %) before adding 2 ml of diethyl ether. The mixture was properly vortexed and then centrifuged at 1700 ×g for 10 min. The diethyl ether (upper) layer of each sample was transferred to a labelled clean glass tube. Ether extract (0.4 ml) and 50 µl N-(tertbutyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, Sigma-Aldrich, Poole, UK) were added into a 2 ml GC screw-cap vial. The mixtures were incubated at room temperature for 72 h to ensure that derivatisation was complete. Standard mixtures containing 5 mM, 2-ethyl butyrate for calibration were extracted and derivatised using the same steps as those for the faecal samples. Subsequently, the samples and standards were analysed using the GC.

Analysis was performed using an Agilent 6890/5975 GC-MS system (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5MS 30 m×0.25 mm column with a 0.25 µm coating

of crosslinked 5% phenyl-methylpolysiloxane, (Hewlett Packard, UK).The carrier gas which was helium had a flow rate of 1.7 ml $min^{-1}$ and head pressure of 113 kPa. The initial temperature was 63 °C and was elevated by 15 $^{o}C$ $min^{-1}$ till it reached 190 $^{o}C$ and then held for 3 min. The injector and detector temperatures were set at 275 $^{o}C$. The test samples were injected (1 µl) with a split ratio of 100:1. Quantification of the samples was achieved by calibration with acetic, propionic, butyric acid with concentration between 25 and 100 mM.

**2.5 Total DNA isolation for enumeration of microbial population using 16s sequencing**

DNA isolation was performed according to the protocol provided by the QIAamp Power Faecal DNA kit (Qiagen, Hilden, and Germany) which was also used for DNA isolation. Briefly, faecal samples obtained from each one of the vessels of the gut model (1 ml) were centrifuged (11337×g for 10 min) and the pellet was re-suspended in 750 µl of power bead solution (QIAGEN). The mixture was added in the dry bead tube provided with the kit and 60 µl solution C1 (QIAGEN) was also added followed by gentle vertexing. Subsequently, the tubes were placed in a water bath at 65 $^{o}C$ for 10 min. The mixture was then vortexed for 10 min and centrifuged at 11337×g for 1 min. The supernatant was transferred to a 2 ml collection tube and 250 µl of solution C2 (QIAGEN) was added followed by brief vortexing. Then the mix was incubated at 4 $^{o}C$ for 5 min and centrifuged at 11337×g for 1 min. Six hundred µl of the supernatant was carefully transferred into a 2 ml collection tube and 200 µl of C3 solution (QIAGEN) was added to the tube and then incubated at 4 $^{o}C$ for another 5 min. Following a centrifugation of the mixture at 11337×g for 1 min, 750 µl of the supernatant was transferred to a clean 2 ml collection tube where 1200 µl of C4 solution (QIAGEN) was added to the supernatant and vortexed for 5 sec. The mixture was then carefully loaded into an MB spin column and centrifuged at 11337×g for 1 min. The flow through was discarded while 500 µl of solution C5 (QIAGEN) was added to the spin column containing the DNA which was centrifuged at 11337×g for 1 min. The spin column was then carefully placed in a

clean 2 ml collection tube and 50 µl of solution C6 (QIAGEN) was added to elute the DNA followed by centrifugation at 11337×g for 1 min. Subsequently, the DNA concentration was assessed using a Nano drop spectrophotometer ND-1000 (Thermo-Fisher, UK) and the DNA was stored at -20 $^{o}$C for further amplification of 16S and sequencing. DNA concentration and purity was assessed on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/µl using sterile water. This step was done by Novogene, Hong Kong.

**2.6 16s bacterial profiling (Novogene, Hong Kong).**

**2.6.1. 16S Gene Amplicon Generation**

| **Gene** | **Region** | **Fragment length** | **Primer** | **Primer sequences ( 5'- 3 )** |
|---|---|---|---|---|
| **Bacterial16S** | **V3-V4** | **466 bp** | **341F** | **CCTAYGGGRBGCASCAG** |
| | | | **806R** | **GGACTACNNGGGTATCTAAT** |

**Table 1:** 16s gene amplicon generation. Showing the region, fragment length, primer with primer sequence.

All PCR reactions were carried out in 30 µL reactions with 15 µL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs) together with 0.2 µM of forward and reverse primers, and about 10 ng templates DNA. Thermal cycling started with an initial denaturation at 98 ℃ for 1 min, followed by 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 60 s and finally at 72 ℃ for further 5 min.

### 2.6.2. PCR Products quantification and qualification

Equal volumes of 1X loading buffer containing SYBR green, was mixed with PCR products and electrophoresis was done on 2% agarose gel for detection. Samples with bright main strip between < 470bp were selected for sequencing.

### 2.6.3. PCR Products Mixing and Purification

PCR products were mixed in equidensity ratios. Then, mixture PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific).

### 2.6.4 Library preparation and sequencing analysis.

Standard bioinformatics analysis such as operational taxonomic units, alpha and beta diversity, species distribution etc. was carried out. Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

# 3. Results

## 3.1 GABA production

The batch culture fermentation carried out in our study simulates the distal colon of the large intestine while proteolysis also takes place in this part of the colon. Supplementing the growth medium with various amino acids showed significant effects on GABA production at different time points (fig1). The pH-controlled fermentation using basal medium showed a significant increase of GABA by up to 8-fold following 48 h fermentation with casamino acids, 6-fold with peptone, and 7–fold with tryptone, 3-fold with L-tryptophan and 4-fold with L-cysteine. Interestingly, yeast extract did not show an effect on GABA levels in comparison with the control without any supplementation (Fig 1A).

Meanwhile in the non-pH-controlled fermentation in BHI (Fig. 1B), there was an increase in GABA production following supplementation with casamino acids (6-fold), yeast extract (5-fold), peptone (5-fold) and tryptone (4-fold) at 24 h with casamino acids showing the most effect with a 3-fold and 6-fold increase at 12 h and 24 h respectively. While L-cysteine showed a significant reduction 3-folds at both 12 and 24 h and L-tryptophan showed a significant reduction of 5- folds and 12-folds at 24 h and 48 h respectively when comparing with the control (without supplementation).

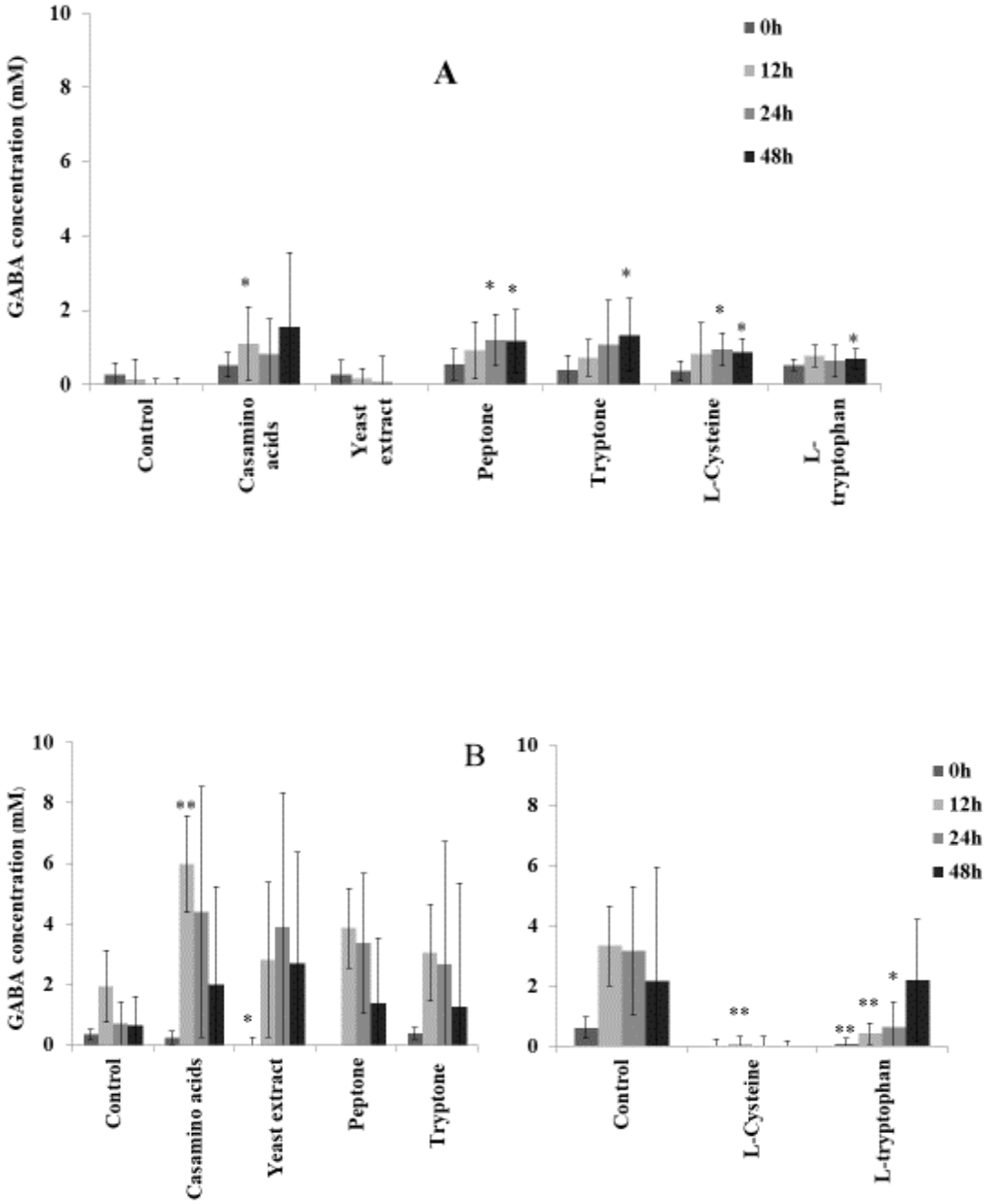


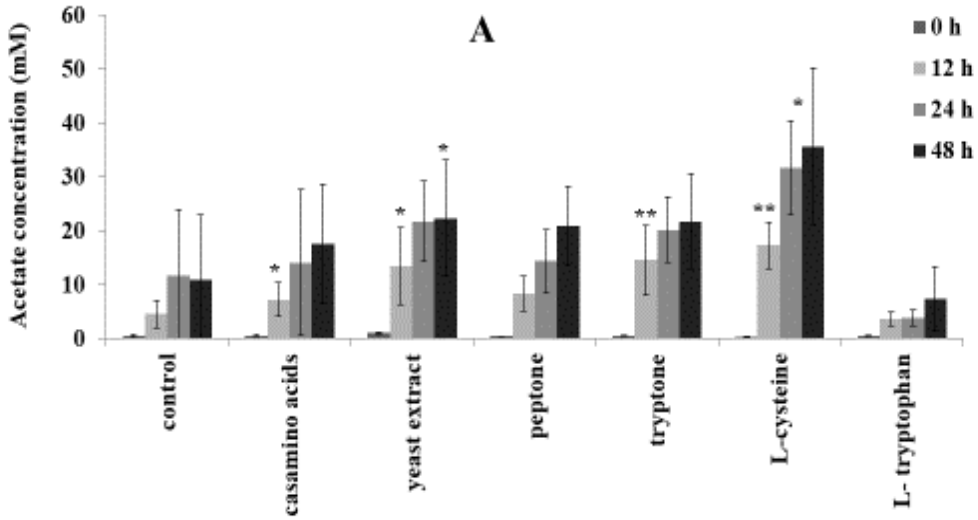

**Fig 1**: GABA concentration (mM) in pH-controlled fermentation using basal media, pH-controlled (A) and in BHI, non-pH-controlled (B) static fermentation in BHI at 37 $^{O}$C for 48 h. Bars represent mean values between 5 donors. Error bars represent standard deviation of the mean values. Asterisk (*) represents mean values that were significantly different from control ($P < 0.05$), and double asterisk (**) representing mean values were highly significantly different from control ($P < 0.01$). Duplicate control values with non-pH-controlled fermentation was as a result of the experiment done twice.

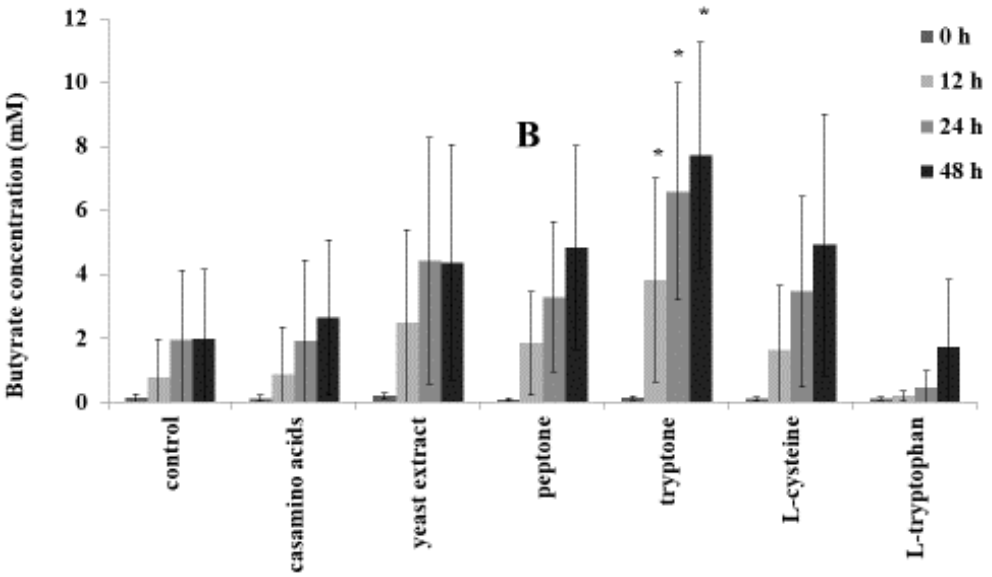
### 3.2 Short chain fatty acid production

#### 3.2.1 pH-controlled batch culture fermentation (Basal medium)

SCFAs such as acetic, butyric and propionic can positively affect the health of both human and animal subjects. Therefore, we looked at the levels of SCFAs in the fermentation vessels.

Acetate concentration was significantly increased following supplementation with casamino acids, yeast extract, peptone, tryptone and L-cysteine with L-cysteine showing the highest increase by 3-fold at both 24 and 48 h when comparing with the control (fig 2A). Also, butyrate concentration was also increased with tryptone being the most effective with an increase of up to 4-fold by the end of the 48 h fermentation (Fig 2B). Likewise, propionate concentration was also raised with yeast extract and tryptone also resulted in a 4-fold increase at the end of the 48 h fermentation (fig 2C). Finally, addition of L-tryptophan did not show any effect on propionic acid production. Meanwhile there was about 1-fold decrease in acetic and butyric acid production with the addition of L-tryptophan (Fig 2).


A
Acetate concentration (mM)
60
50
40
30
20
10
0
0 h
12 h
24 h
48 h
control
casamino acids
yeast extract
peptone
tryptone
L-cysteine
L- tryptophan


B
Butyrate concentration (mM)
12
10
8
6
4
2
0
0 h
12 h
24 h
48 h
control
casamino acids
yeast extract
peptone
tryptone
L-cysteine
L-tryptophan

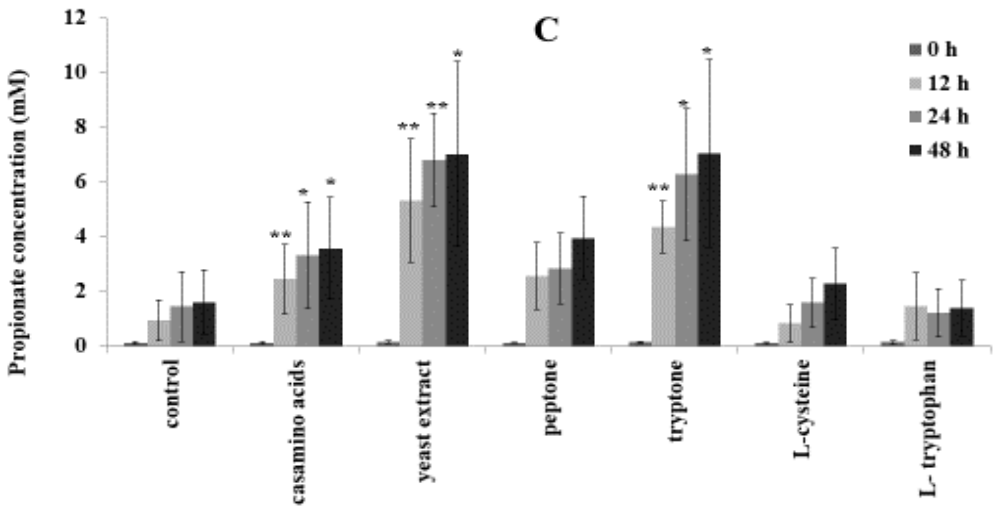



**Fig 2:** SCFA levels, namely acetate (A), butyrate (B) and propionate (C) following supplementation with amino acid and peptide sources, or without supplementation (control) in a pH-controlled single stage culture fermentation in basal media 37 $^{o}$C for 48 h. Bars represent mean values between 5 donors while error bars represent standard deviation. Asterisk (*) represent statistically significant difference ($P < 0.05$), double asterisk (**) represents very statistically significant different ($P < 0.01$) and triple asterisk (***) represents highly statistically different ($P < 0.001$) from the control (without supplementation)

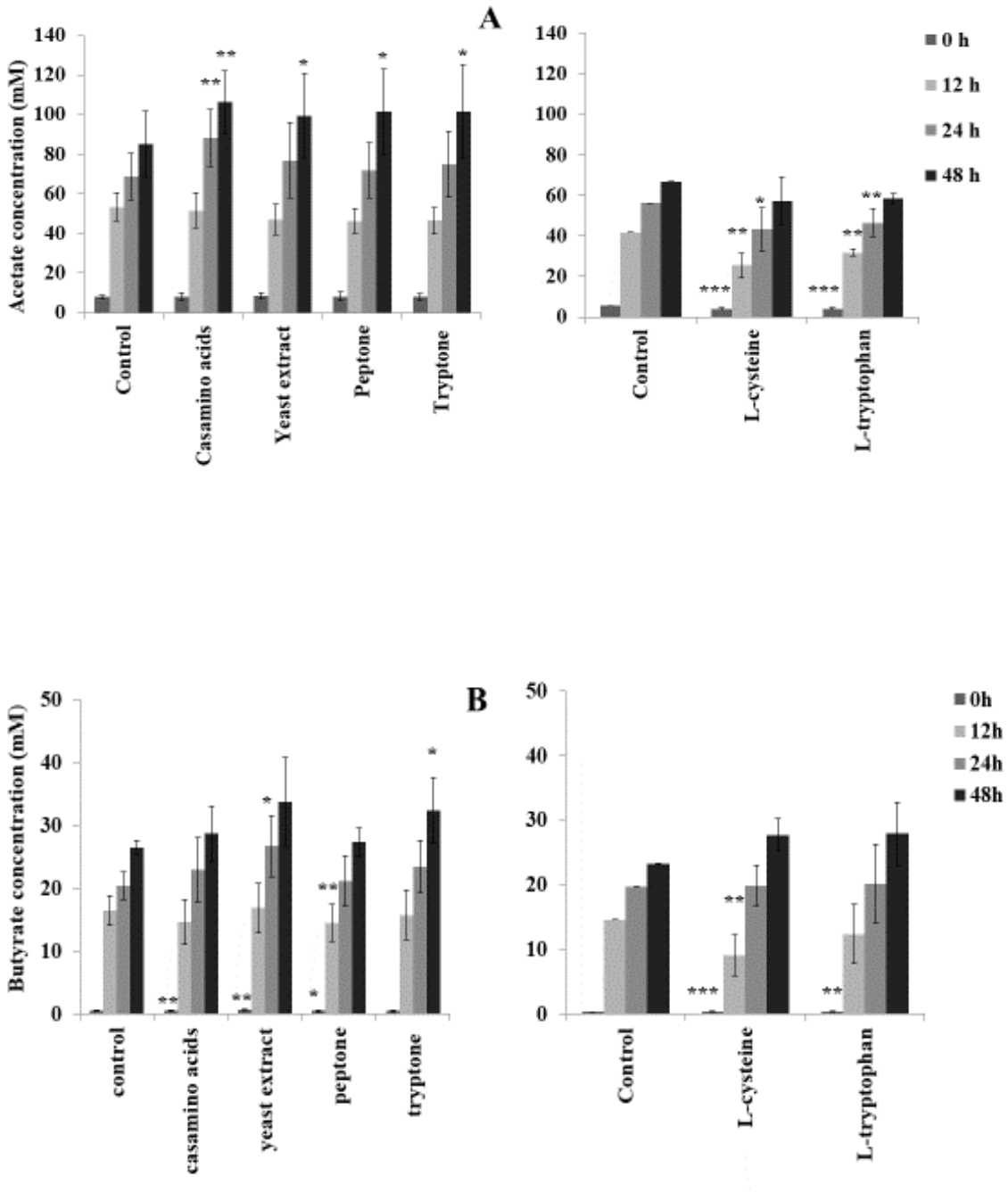
.

### 3.2.2. Static (non-pH) controlled fermentation (BHI)

The supplementation with casamino acids, yeast extract, peptone and tryptone showed a significant increase with acetic and butyric acid production at various time points with the exception of propionic acid where the increase was not significant. In contrast, L-cysteine and L-tryptophan showed a significant decrease in the concentration of all three acids (fig 3).

There was a 1-fold increase in acetic acid at 48 h with the addition of casamino acids, yeast extract, peptone and tryptone (Fig. 3A). Although significant, L-cysteine and L-tryptophan showed less than 1-fold decrease at 0, 12, 24 and 48 h (Fig. 3A). Also, there was approximately 1-fold increase in butyric acid with the addition of all amino acids (Fig 3B). Furthermore, there

was little or no effect on the concentration of propionic acid with the amino acids except for L-cysteine which significantly reduced propionic acid concentration by fold at 12 and 48 h (fig 3c).



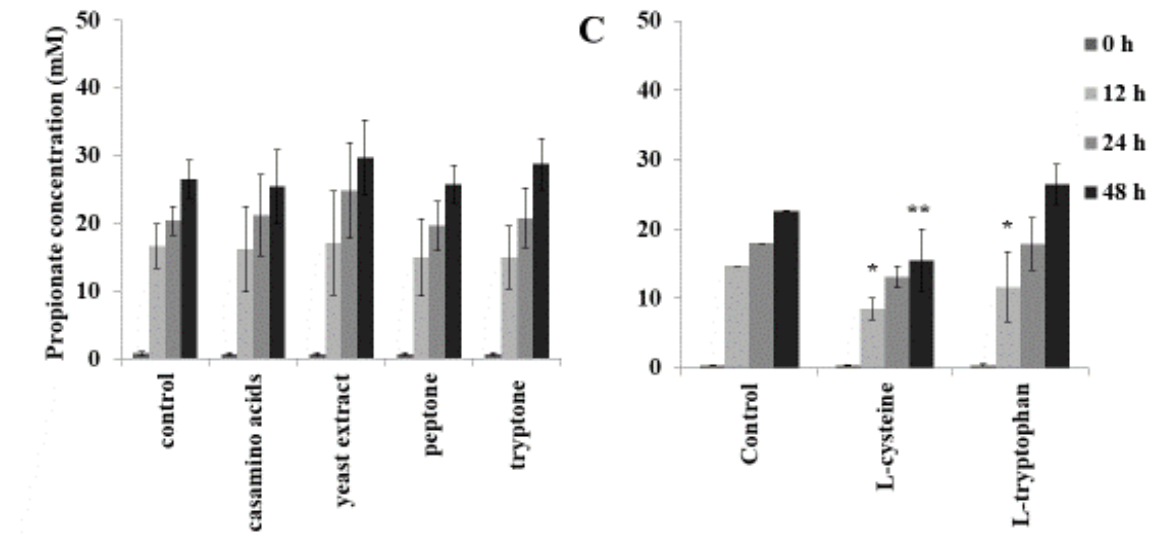



**Fig 3:** SCFA production, namely acetate (A) butyrate (B) and propionate (C) in non-pH controlled static batch culture fermentation system in BHI. Bars are mean values between 5 donors. Error bars represents standard deviation. Asterick * Mean values were significantly different from SS1 ($P < 0.05$), ** Mean values were highly significantly different from control ($P < 0.01$) and *** Mean values were very highly significantly different from control ($P < 0.001$). Dublicate controls was due to the fact that experiments were performed twice with the different supplements.

### 3.3 Bacterial 16S sequencing showing top 10 most abundant genus

#### 3.3.1 pH-controlled batch culture fermentation (basal medium)

There was a 1-fold decrease in *Escherichia-Shigella* with the addition of casamino acids, yeast extract, peptone, tryptone and a 1-fold increase in *Escherichia-Shigella* with the addition of L-cysteine and L-tryptophan when comparing with control at 24 h. Also, there was an increase in *Acidaminococcus* with the addition of yeast extract (8-fold), peptone (29-fold), tryptone (20-fold) and L-cysteine (12-fold) when comparing with the control at 24 h (Fig 4).

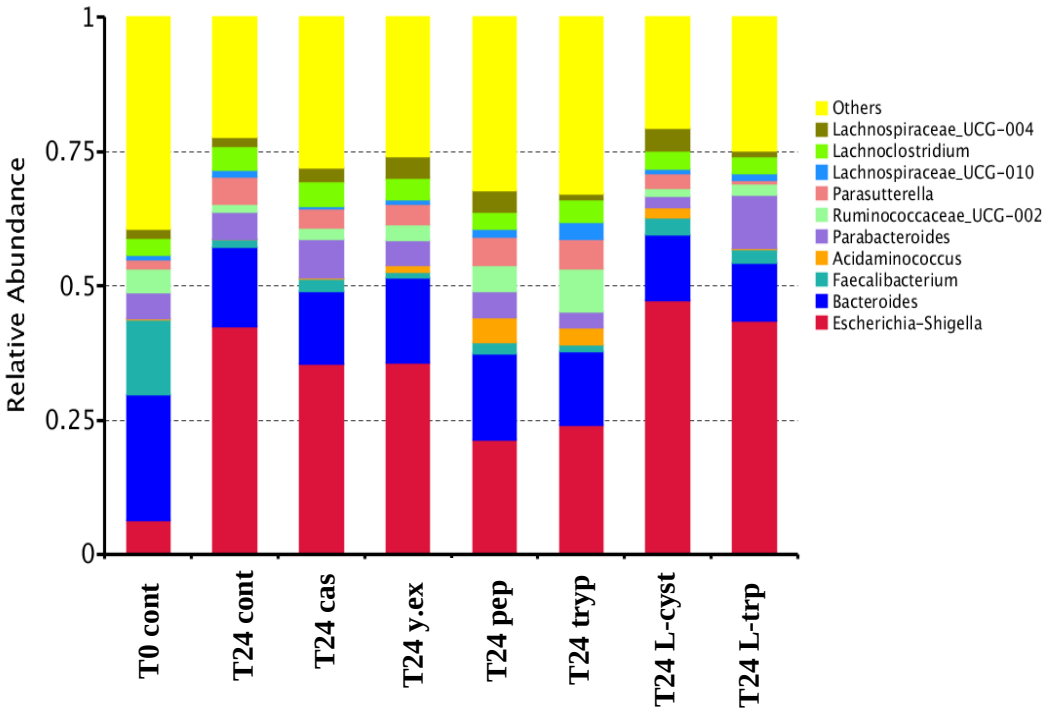



**Fig 4:** Operational taxonomic unit (OTU) analysis for the top 10 genera based on relative abundance from the 16S RNA sequencing of samples taken from pH-controlled batch culture fermentation. Where T0 cont: control at 0 h, T24 cont: control at 24 h, T24 cas: casamino acids supplemented at 24 h, T24 pep: peptone supplemented at 24 h, T24 tryp: tryptone supplemented at 24 h, T24 cys : L-cysteine supplemented at 24 h and T24 Trp: L-trypthophan supplemented at 24 h. The control represents samples without supplementation.

### 3.3.1 Static (non-pH) controlled fermentation (BHI)

There was 1-fold decrease in *Prevotella, Feacalibacterium and Bacteroides* and a 2-fold increase in *Bifidobacterium* with the addition of L-tryptophan and L-cysteine. Meanwhile with the addition of casamino acids, yeast extract, peptone and tryptone there was 1-fold increase in *Lachnoclostridium* and a reduction (< 1-fold) in *Prevotella* when comparing with control at 24 h.

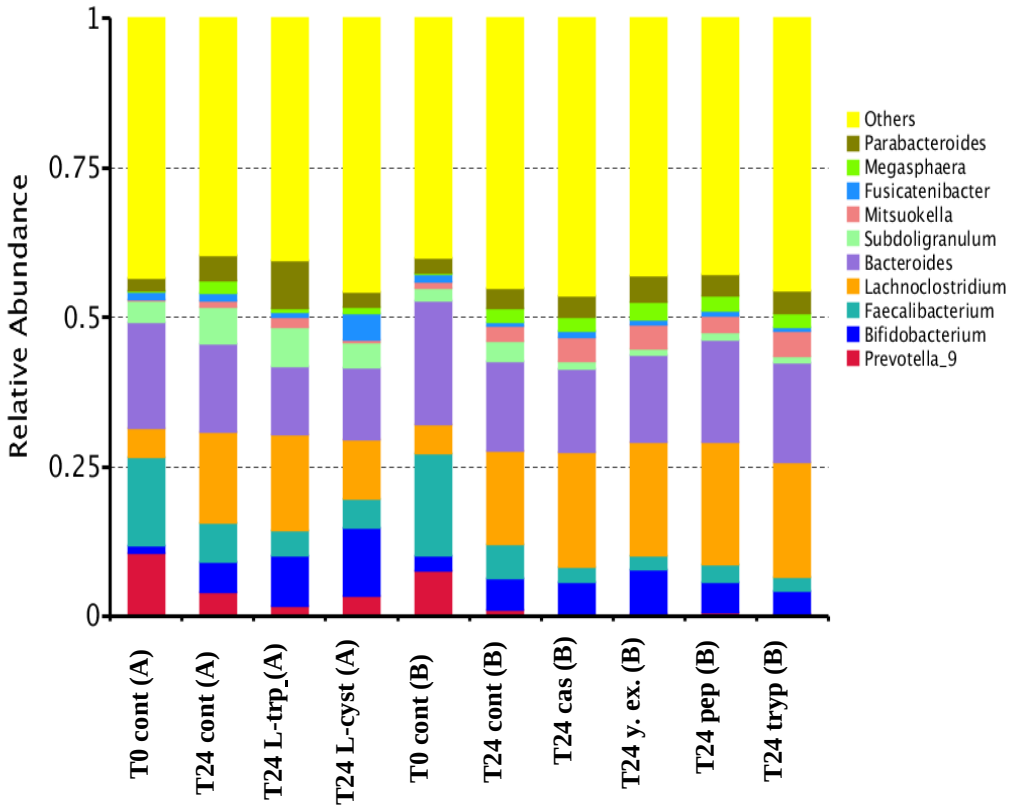



**Fig 5:**Operational taxonomic unit (OTU) analysis for the top 10 genera based on relative abundance from the 16S RNA sequencing of samples taken from non-pH-controlled batch culture fermentation. A represents for the first set of fermentation experiments while B is the second set. Where T0 con: is control at 0h, t24 con: control at 24 h, T24 L-trp: L-tryptophan supplemented at 24 h, T24 L-cyst: L-cysteine supplemented at 24 h, T24 cas: casamino acids supplemented at 24 h, T24 y.ex.: yeast extract supplemented at 24 h, T24 pep: peptone supplemented at 24 h and T24 tryp: tryptone supplemented at 24 h. The control represents samples without supplementation.

### 3.4 SPSS (Pearson correlation) analysis correlating bacterial profiling with GABA, Acetate, butyrate and propionate production.

Pearson correlation was performed in both the pH-controlled and non-pH-controlled fermentation but at the genus level we did not find any significant correlation with the microbial profile which could possibly suggest that the metabolic activity occurring during the fermentation were more species specific as there were significant correlations at the species level. The analysis showed a positive correlation with some bacterial genera while a larger percentage of the identified genera showed a negative correlation especially with the production of SCFAs which means that a reduction in the population of these genera leads to an increase in SCFAs concentration (Table 2).

| **Pearsons Correlations (N= 50)** | **(A) GABA** | **Pearsons Correlations (N= 50)** | **(B) Acetate** | **Pearsons Correlations (N= 50)** | **(C) Butyrate** | **Pearsons Correlations (N= 50)** | **(D) Propionate** |
|---|---|---|---|---|---|---|---|
| *Mitsuokella* | 0.533** | Propionate | 0.852** | *Propionate* | 0.836** | Acetate | 0.852** |
| *Megasphaera* | 0.520** | Butyrate | 0.762** | *Acetate* | 0.762** | Butyrate | 0.836** |
| *Peptoniphilus* | 0.485** | *Dorea* | 0.752** | *Lachnoclostridium* | 0.526** | *Dorea* | 0.566** |
| *Succiniclasticum* | 0.485** | *Erysipelatoclostridium* | 0.603** | *Dorea* | 0.503** | *Candidatus_Soleaferrea* | 0.488** |
| *Murdochiella* | 0.474** | *Candidatus_Soleaferrea* | 0.602** | GABA | 0.417** | *Dialister* | 0.461** |
| *Negativicoccus* | 0.473** | *Clostridium_innocuum_group* | 0.587** | *Megasphaera* | 0.406** | GABA | 0.436** |
| *Olsenella* | 0.451** | *Lachnoclostridium* | 0.585** | *Mitsuokella* | 0.393** | *Lachnoclostridium* | 0.416** |
| *Propionate* | 0.436** | *Anaerotruncus* | 0.532** | *Peptoniphilus* | 0.371** | *Hungatella* | 0.408** |
| *Butyrate* | 0.417** | *Ruminococcus_gnavus_group* | 0.520** | *Enterococcus* | 0.370** | *Veillonella* | 0.391** |
| *Senegalimassilia* | 0.374** | *Escherichia_Shigella* | 0.514** | *Olsenella* | 0.369** | *Coprococcus_3* | 0.386** |
| *Enterococcus* | 0.368** | *Eggerthella* | 0.505** | *Pyramidobacter* | 0.365** | *Anaerostipes* | 0.383** |
| *Veillonella* | 0.367** | *Hungatella* | 0.495** | *Hungatella* | 0.363** | *Sutterella* | 0.383** |
| *unidentified_Family_XIII* | 0.346* | *unidentified_Erysipelotrichaceae* | 0.482** | *Murdochiella* | 0.348* | *Clostridium_innocuum_group* | 0.378** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Prevotella_7* | 0.341* | *Pseudoflavonifractor* | 0.466** | *Negativicoccus* | 0.346* | *Eggerthella* | 0.370** |
| *Dialister* | 0.322* | *Flavonifractor* | 0.464** | *unidentified_Family_XIII* | 0.344* | *Pseudoflavonifractor* | 0.348* |
| *Coprococcus_1* | 0.315* | *Eubacterium_nodatum_group* | 0.450** | *Collinsella* | 0.337* | *Gordonibacter* | 0.346* |
| *Porphyromonas* | 0.305* | *Eubacterium_hallii_group* | 0.448** | *Acidaminococcus* | 0.322* | *Flavonifractor* | 0.323* |
| *Acinetobacter* | 0.304* | *Gordonibacter* | 0.425** | *Parabacteroides* | 0.319* | *Paeniclostridium* | 0.318* |
| *Hungatella* | 0.298* | *Intestinimonas* | 0.422** | *Sutterella* | 0.299* | *Erysipelatoclostridium* | 0.314* |
| *Holdemanella* | -0.288* | *Blautia* | 0.407** | *Dielma* | 0.285* | *Intestinimonas* | 0.308* |
| *Butyricimonas* | -0.289* | *Lactonifactor* | 0.397** | *Succiniclasticum* | 0.279* | *unidentified_Erysipelotrichaceae* | 0.305* |
| *Anaerotruncus* | -0.292* | *Sellimonas* | 0.384** | *Lachnospiraceae_UCG_003* | -0.298* | *Escherichia_Shigella* | 0.284* |
| *Ruminiclostridium_9* | -0.294* | *Family_XIII_AD3011_group* | 0.367** | *Christensenellaceae_R_7_group* | -0.299* | *Sellimonas* | 0.279* |
| *Acetanaerobacterium* | -0.294* | *Coprococcus_3* | 0.367** | *unidentified_Gastranaerophilales* | -0.306* | *Lachnospiraceae_UCG_003* | -0.291* |
| *Erysipelatoclostridium* | -0.297* | *Senegalimassilia* | 0.365** | *Pseudobutyrivibrio* | -0.307* | *Ruminiclostridium_5* | -0.298* |
| *Ruminococcus_1* | -0.298* | *Ruminococcaceae_UCG_004* | 0.363** | *Coprococcus_2* | -0.310* | *Christensenellaceae_R_7_group* | -0.302* |
| *Eubacterium_ventriosum_group* | -0.327* | *Coriobacteriaceae_UCG_002* | 0.346* | *Lachnospiraceae_UCG_010* | -0.315* | *Barnesiella* | -0.304* |
| *Ruminiclostridium_5* | -0.366** | *Holdemania* | 0.324* | *Prevotella_6* | -0.316* | *Pseudobutyrivibrio* | -0.321* |
| *Blautia* | -0.413** | *Sutterella* | 0.295* | *Prevotella_9* | -0.325* | *Lachnospiraceae_UCG_010* | -0.327* |
| | | *Anaerostipes* | 0.290* | *Coprobacter* | -0.330* | *Eubacterium_ruminantium_group* | -0.329* |
| | | *Dielma* | 0.289* | *Gelria* | -0.332* | *Haemophilus* | -0.329* |
| | | *Ruminiclostridium_9* | 0.287* | *Ruminococcaceae_UCG_010* | -0.333* | *Peptococcus* | -0.334* |
| | | *Alistipes* | -0.285* | *Lachnospiraceae_FCS020_group* | -0.334* | *Ruminococcaceae_UCG_007* | -0.335* |
| | | *Eubacterium_ruminantium_group* | -0.285* | *Ruminiclostridium_6* | -0.335* | *Turicibacter* | -0.339* |
| | | *Gelria* | -0.288* | *Haemophilus* | -0.335* | *Prevotella_9* | -0.345* |
| | | *Rikenellaceae_RC9_gut_group* | -0.288* | *Ruminiclostridium_5* | -0.350* | *Oxalobacter* | -0.354* |
| | | *Ruminococcus_1* | -0.288* | *Variovorax* | -0.372** | *Bilophila* | -0.355* |
| | | *Coprobacter* | -0.292* | *Oxalobacter* | -0.375** | *unidentified_Gastranaerophilales* | -0.358* |
| | | *Prevotella_6* | -0.294* | *Ruminococcaceae_UCG_014* | -0.383** | *Variovorax* | -0.360* |
| | | *Turicibacter* | -0.306* | *Paraprevotella* | -0.393** | *Ruminiclostridium_6* | -0.362** |
| | | *Eubacterium_ventriosum_group* | -0.312* | *Eubacterium_ventriosum_group* | -0.410** | *Gelria* | -0.368** |
| | | *Haemophilus* | -0.315* | *Romboutsia* | -0.414** | *Alistipes* | -0.373** |
| | | *Rothia* | -0.316* | *Oribacterium* | -0.415** | *Oribacterium* | -0.377** |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | *unidentified_Gastranaerophilales* | -0.320* | *Ruminococcaceae_UCG_013* | -0.424** | *Coprobacter* | -0.382** |
| | | *Lachnospiraceae_UCG_010* | -0.331* | *Peptococcus* | -0.428** | *Lachnospiraceae_ND3007_group* | -0.384** |
| | | *Oribacterium* | -0.336* | *Ruminococcaceae_NK4A214_group* | -0.499** | *Rothia* | -0.394** |
| | | *Variovorax* | -0.339* | *Bilophila* | -0.452** | *Ruminococcus_1* | -0.399** |
| | | *Pseudobutyrivibrio* | -0.345* | *Eubacterium_xylanophilum_group* | -0.458** | *Ruminococcaceae_NK4A214_group* | -0.419** |
| | | *Prevotella_9* | -0.349* | *Roseburia* | -0.460** | *Eubacterium_xylanophilum_group* | -0.430** |
| | | *Lachnospiraceae_FCS020_group* | -0.350* | *Turicibacter* | -0.449** | *Ruminococcaceae_UCG_010* | -0.437** |
| | | *Ruminococcaceae_UCG_013* | -0.357* | *Family_XIII_UCG_001* | -0.532** | *Ruminococcaceae_UCG_013* | -0.444** |
| | | *Peptococcus* | -0.366** | *Alistipes* | -0.539** | *Family_XIII_UCG_001* | -0.448** |
| | | *Family_XIII_UCG_001* | -0.379** | *Eubacterium_coprostanoligenes_group* | -0.544** | *Paraprevotella* | -0.459** |
| | | *Oxalobacter* | -0.381** | *Ruminococcaceae_UCG_005* | -0.553** | *Romboutsia* | -0.473** |
| | | *Romboutsia* | -0.395** | *Ruminococcus_1* | -0.556** | *Eubacterium_ventriosum_group* | -0.477** |
| | | *Ruminococcaceae_NK4A214_group* | -0.396** | *Eubacterium_rectale_group* | -0.569** | *Eubacterium_coprostanoligenes_group* | -0.526** |
| | | *Lachnospiraceae_ND3007_group* | -0.420** | *Lachnospiraceae_ND3007_group* | -0.599** | *Lachnospira* | -0.528** |
| | | *Eubacterium_xylanophilum_group* | -0.432** | *Lachnospira* | -0.629** | *Roseburia* | -0.544** |
| | | *Paraprevotella* | -0.436** | *Eubacterium_eligens_group* | -0.676** | *Ruminococcaceae_UCG_005* | -0.577** |
| | | *Eubacterium_coprostanoligenes_group* | -0.499** | *Faecalibacterium* | -0.754** | *Eubacterium_eligens_group* | -0.627** |
| | | *Ruminococcaceae_UCG_005* | -0.516** | | | *Eubacterium_rectale_group* | -0.641** |
| | | *Lachnospira* | -0.523** | | | *Faecalibacterium* | -0.660** |
| | | *Roseburia* | -0.555** | | | | |
| | | *Eubacterium_rectale_group* | -0.577** | | | | |

**Table 2:** Pearson correlation at genus level: (A), (B), (C) and (D) is the correlation between bacterial genera profile and GABA, acetate, butyrate and propionate levels respectively in the non-pH controlled fermentation. Asterisk (*) indicates significant correlation (P<0.05; 2-tailed) and double asterisk (**) indicates very significant correlation (P<0.01; 2-tailed) and N which is the sample size = 50

# 4. Discussion

It has been estimated that about 3-12 gr of dietary protein and peptides reach the large intestine on a daily basis and these dietary protein and peptides serve as a source of nitrogen to the gut microbiota. Bacterial growth in the gut link with carbohydrate fermentation but it is majorly stimulated by peptides and amino acids present (Argyle and Baldwin, 1989). There has been no previous study to show the effect of amino acids such has casamino acids, tryptone, peptone etc. on GABA production in the gut microbiota. Therefore, the purpose of this study, was to investigate the impact of casamino acids, yeast extract, peptone, tryptone, L-cysteine and L-tryptophan on the make-up of the gut microbiota, the GABA and SCFA production in the gut.

GABA is a non-protein amino acid and the major inhibitory neurotransmitter of the central nervous system of mammals (Watanabe et al., 2006, Tsukatani et al., 2005, Barrett et al., 2012). Its production is the result of microbial fermentation as when fermentation occurs there is a drop in the extracellular and consequently in the intracellular pH of the microbial cells which activates the glutamate decarboxylase (GAD) system resulting in the production of GABA (Dhakal et al., 2012).

The microbial GABA production is influenced by various factors such as pH, temperature, medium composition and fermentation time. Scientists have been working on optimizing GABA production of individual microbial strains isolated from fermented products or in the fermented products themselves. For instance, several studies have been conducted on optimizing GABA production in several *Lactobacillus* species isolated from fermented foods such as cheeses, kimchi etc (Lim et al., 2017, Siragusa et al., 2007a) .

Culture conditions such as medium components especially carbon and nitrogen sources significantly influence microbial GABA production (Lim et al., 2017). In this study we looked

at optimizing GABA production in the gut microbiome by supplementing the culture media with amino acids or mixes of aminoacids and peptides which serve as nitrogen source. The amino acids are utilized as a source of energy by the gut microbiota and metabolic end products such as SCFAs and GABA can help the bacteria in adapting to the stress conditions in the gut. Therefore, we found (Fig. 1) that the addition of amino acids/ peptides such as casamino acids, tryptone and peptone increase the production of GABA in the vessels which suggests that intestinal bacteria were able to utilize these amino acids during fermentation to influence GAD activity and hence improve GABA production. Interestingly, there was a higher concentration of GABA produced with the non-pH controlled fermentation and this is supporting the idea that at low pH conditions more GABA is produced as the bacterial GAD system is activated at a low pH (Feehily and Karatzas, 2013). Although, the non-pH-controlled fermentation was carried out in BHI medium.

SCFA production is dependent on the host intestinal environmental conditions, dietary and microbiological factors. Therefore, the availability of substrates largely determines the amount and the type of SCFA that will be produced (Macfarlane and Macfarlane, 2007). There was an increase in the production of acetate, butyrate and propionate by supplementing with casamino acids, peptone, tryptone, yeast extract and L-cysteine except for L-tryptophan in the pH-controlled fermentation (Fig. 2) while in the non- pH-controlled fermentation (Fig. 3) there was a significant reduction of acetate with L-cysteine as well as L-tryptophan. Also, the production of acetate, butyrate and propionate with non-pH-controlled fermentation was higher than the pH-controlled and it can be deduced from this results that SCFA pools were higher at lower pH during protein fermentation. Although, the fermentations were carried out in separate growth media so the comparisons between both batch culture fermentation could be indication of our findings.

Although, from this study there was vast difference in the effects of supplementing the growth media with amino acids in the pH-controlled and non-pH-controlled fermentation. This difference could possibly be linked to the culture conditions in which we found higher levels of GABA and SCFA concentration in the non-pH-controlled fermentation with lower pH values than the pH-controlled fermentation. The increased levels of GABA and SCFA in the non-pH-controlled fermentation could possibly be linked to the drop in pH GABA is produced at a lower pH.

Also, it is important to highlight the fact that media composition also plays an important role in the production of these metabolites as the pH-controlled fermentation was carried out in basal medium which is a basic medium with no nutrients to support the production of these metabolites while the non-pH-controlled fermentation was conducted in brain heart infusion broth (BHI) which is a rich growth medium.

Although basal medium is a non-supplemented growth media used in batch culture fermentation, BHI is a nutrient rich growth medium that contains some source of amino acids. Therefore, it could be suggested that the extra source of amino acid in BHI is responsible for these higher levels of metabolites produced. Also, previous experiments from our LAB has shown that Chemically Defined Medium does not support the production of GABA compared to using a nutrient rich culture media such as BHI (Karatzas et al., 2010).

Several species of bacteria have the potential of utilizing amino acids as substrates for their growth. Although *Clostridıum* group are more dominant during amino acids fermentation in the human colon, other species such as *Fusobacterium, Bacteroides, Veillonella, Megasphaera* and *Selenomonas* are also important in metabolizing amino acids (Dai et al., 2011, Smith and Macfarlane, 1998). Interestingly, our results (Fig 4 & 5), show that some of these bacteria (e.g. *Bacteriodes, Megasphaera, Lachnoclostridium* and *Parabaceteroides*) that utilise amino acids

were in the top ten of the most abundant genera found with OTU analysis (16S sequencing) during the batch culture fermentation.

The Pearson correlation using SPSS showed no significant correlation GABA and SCFA produced with any of the genera of the microbial profile in the pH-controlled fermentation compared to the non-pH-controlled fermentation. Although, there was a correlation at the species level with the microbial profile and GABA/SCFA acid produced. Therefore, it can be inferred from these findings that the specific metabolic activities occur at the genus level are completely different as a result of the difference in environmental and culture conditions. It could also be suggested that the metabolic activity in the pH-controlled fermentation is more species-specific. Some of the GABA-producing strains isolated from the gut have been identified by (Strandwitz et al., 2018) and (Barrett et al., 2012) and these include *Bacteroides, Lactobacillus, Megasphaera, Bifidobacteria, Alistipes, Parabacteroides, Clostridum, Eubacterium* and *Fusobacterium.* These bacterial strains including *Prevotella, Esherichia, Enterococcus* and *Acinetobacter have* been reported to have the GAD system that enables them to produce GABA. Therefore, some of the genera like *Megasphaera, Actinetobacter, Prevotell*a can be seen in the Pearson correlation of the non-pH controlled fermentation to have a positive correlation with GABA production in our study.

Furthermore, acetate-producing bacteria such as *Eubacterium, Clostridium* and *Ruminococcus,* butyrate-producing bacteria such as *Peptoniphilus* and *Clostridium* and propionate-producing bacteria like *Dialister, Veillonella* and *Clostridium* were identified from the test as having positive correlation to the metabolites produced i.e. acetate, butyrate and propionate respectively.

Finally, batch culture fermentation is a fast technique in analysing the impact of various amino acid on the gut microbiota but the fermentation system is quite limiting as SCFAs would be

absorbed in the human colon and also the supply of food to the colon is a continuous process. These has led us into the study of the effect of selected amino acids in the gut model which is a broader way of simulating the human colon.

## 5. Conclusions

In conclusion, bacterial growth in the gut is associated with carbohydrate fermentation but it is majorly stimulated by peptides and amino acids present. Amino acids are good nitrogen sources in the gut therefore, their supplementation in the diet (milk, yoghurt, eggs etc.) could possibly increase the production of GABA, SCFA which are metabolites produced during bacterial fermentation in the gut. Furthermore, we found that culture conditions and media used also influence the production of GABA and SCFA in the gut. Therefore, increasing the production of these metabolites (GABA and SCFAs) in the gut can be of major health benefit to the human body and also advances in the knowledge of food components or supplements could give a better understanding of its effects on the gut and the gut microbiota. Such research could also help us understand possible negative effects of the gut microbiota but also result in possible interventions that through diet increase the levels of GABA and SCFAs in the gut.

## 6. Acknowledgements

The authors would like to thank all colleagues at the Microbiology research team, Food and Nutritional Sciences, University of Reading, Centre for Integrative Neurosciences and Neurodynamics (CINN) for the grant given towards the sequencing, Society for Applied Microbiology (SFAM) for the studentship grant given and Novogene, Hong-Kong for 16S bacterial profiling including the library preparation, sequencing and bioinformatics analysis.

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# Chapter 5

# Effect of casaminoacids and L-cysteine on γ-aminobutyric acid (GABA) and short chain fatty acid (SCFA) production in the gut in a multiple stage continuous culture fermentation model

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# Abstract

γ-Aminobutyric acid (GABA), a major inhibitory neurotransmitter of the central nervous system and has been studied due to its important health benefits. Most studies until now have focused on orally consumed GABA and not so much on GABA produced by the gut microbiota. This study focuses on GABA production from gut microbiota and how environmental conditions can affect it. In previous work, we found that the use of amino acids and peptides boosted the production of GABA in defined medium in a simple one-stage model. Therefore, we decided to investigate the effects of casamino acids and L-cysteine on GABA and short chain fatty acids (SCFAs) production in a three-stage continuous culture fermentation model that simulates the proximal, transverse and distal colon. We found a significant increase in GABA production at steady state (SS) 2 in the proximal, transverse and distal colon with the addition of both casamino acids and L-cysteine. We also found that L-cysteine resulted in an increase in acetate at the transverse and distal colon and a significant decrease in butyrate in the proximal and distal colon. On the other hand, casamino acids resulted in a significant decrease in propionate in all vessels and a significant increase in butyrate in the transverse and distal colon. In conclusion, ingestion of amino acid-rich foods may increase the GABA and SCFA production in the colon by gut bacteria. Therefore, the study of the production of these metabolites in the gut can possibly be a link between the diet, gut microbiota and human physiology.

# 1. Introduction

The gut is an important organ that is colonized by a very complex community of microorganisms which helps in the digestion, absorption and metabolism of various dietary nutrients (Wang et al., 2009). The metabolic function of the gut is dependent on the wide variety of substrates available during fermentation in the colon. Therefore, the metabolites produced by the gut microbiota are absorbed and used up by the host and thus could influence health either positively and negatively. Thus, the link between these fermentation substrates, host and the gut microbiota is essential for maintaining a balance in the ecosystem while a shift in this balance can affect the functionality of gut microbiota hence, leading to dysbiosis (Bernalier-Donadille, 2010).

In recent years, there has been a growing interest in the physiological and nutritional effects of amino acids on the health, growth, survival and the progress of diseases of both humans and animals. Amino acids are building blocks of protein and are important substrates for the synthesis of low molecular weight compounds such as glutathione, polyamines, serotonin nitric oxides e.t.c. They are essential for the regulation of major metabolic pathways to improve the health of the host (Wu, 2009, Wu, 2013). Studies have shown that supplementing diet with amino acids modulates gene expression, enhances bacterial growth in the small intestine, enhances growth of skeletal muscles, reduces excessive body fat, regulates neurological development and function, stimulates protein synthesis and inhibits intracellular proteolysis etc. (Wu, 2013). For instance, (Bannai and Kawai, 2012) have shown that the use of orally ingested glycine improves neurological functions and sleep quality in both human and rat models. Also, dietary supplementation with arginine increased the growth of skeletal muscles in neonatal pigs (Yao et al., 2008). Jobgen et al., (2009) also have shown that dietary supplementation of rats with arginine enhances the expression of major genes that promotes

lipolysis, oxidation of energy substrates and removal of oxidants. By looking at gene expression they were able to conclude that supplementation with arginine is beneficial in reducing the gain of white fat (white adipose tissue), improving insulin sensitivity and anti-oxidative defence capacity in mammals. Therefore, based on these observations and previous work in our lab, we decided to explore the effects of casamino acids and L-cysteine on the production of γ-aminobutyric acid (GABA) and short chain fatty acid (SCFA) by the gut microbiota using the three-stage continuous culture fermentation model simulating the proximal, transverse and distal colon in the large intestine of the human subject.

GABA and SCFA are both metabolites produced by the intestinal microbiota that have a beneficial effect on the health of the host. GABA is a major inhibitory neurotransmitter of the central nervous system of mammals. It is produced during the irreversible decarboxylation of glutamate by the glutamate decarboxylase (GAD) system (Feehily and Karatzas, 2013). GABA is an inhibitory neurotransmitter by providing a relaxing effect on users by preventing the nerve cells in the brain from receiving a stimulatory effect (Harris and Allan, 1985). Studies on GABA have shown that it can reduce the blood pressure in hypertensive animal and human models (Hayakawa et al., 2004, Inoue et al., 2003, Tanaka et al., 2009). It has also been shown to prevent sleep disorders, mood disorders and depression (Ting Wong et al., 2003, Sasaki et al., 2006, Krystal et al., 2002, Bjork et al., 2001). SCFA on the other hand are the primary end products of fermentation of non-digestable carbohydrates that become available to the gut microbiota. They are produced mainly via the saccarolytic fermentation of carbohydrates that are not digested or absorbed in the small intestine (Morrison and Preston, 2016). Amino acid fermentation may also contribute to the production of SCFAs of which the major ones are acetate, propionate and butyrate that are normally present in the colon in a molar ratio of 60:20:20 (Morrison and Preston, 2016, den Besten et al., 2013). Studies have shown that SCFA play an essential role in the prevention of metabolic syndrome

and bowel disorders. Butyrate has been shown to inhibit inflammation and carcinogenesis in the human colon (Hu et al., 2010, HAMER et al., 2008). Also, clinical studies have shown that administered SCFA influence the treatment of diseases such as Crohn's disease, ulcerative colitis and diarrhea (Binder, 2010, Harig et al., 1989, SABATINO et al., 2005). The aim of our study was to determine the effect of casamino acids and L-cysteine on the production of GABA and SCFA in the gut and also possibly identify the bacteria responsible for the increase in production of these metabolites or the possible microbiota changes that accompany these effects.

# 2. Materials and methods

### 2.1 Three-stage continuous culture fermentation model

The three-stage continuous fermentation culture system was used to simulate the luminal conditions in each of the three distinct regions of the human colon: proximal (V1), transverse (V2), and distal colon (V3) (Macfarlane et al., 1998) .Vessels V1, V2 and V3 with an operating volume of 80, 100 and 120 ml were set up sequentially to represent the proximal, transverse and distal colon respectively. Autoclaved culture medium (51.43 ml for 1, 66.67 ml for V2, 82.5 ml for V3) was aseptically poured into the sterile vessels. The system was left overnight with oxygen-free nitrogen pumping through the media at a rate of 15 mL/min. Each vessel was temperature controlled at 37°C using a water bath and stirred using a magnetic stirrer. Faecal slurry at 20% (w/v) was inoculated into the culture vessels (28.57 ml (V1), 33.33 ml (V2), 37.5 ml (V3)) and was left to equilibrate for 24 h as a batch culture system prior to commencing the continuous medium flow. Control of pH was achieved by pH probes - controllers (Electro lab pH controller, Tewksbury, UK) connected to each vessel to regulate the pH at 5.4-5.6 in V1 (proximal colon), 6.1-6.3 in V2 (transverse colon) and 6.7- 6.9 in V3 (distal colon) with the use of 0.5 M HCl and NaOH. Each vessel was magnetically stirred and

anaerobic conditions were maintained throughout the experiment. A sterile 5 L growth/gut model medium was continuously sparged with oxygen free nitrogen and was fed into V1 using a peristaltic pump which was then sequentially supplied to V2 and V3. The culture medium was prepared in sterile distilled water and contained 5.0 g/l starch, 2.0 g/l pectin from citrus 1.0 g/l guar gum, 4.0 g/l mucin (porcine gastric type III), 2.0 g/l xylan from beech wood pure (SERVA, Heidelberg, Germany), 2.0 g/l arabinogalactan from larch wood 1.0 g/l inulin (BENEO-Orafti, Tienen, Belgium), 3.0 g/l casein, 5.0 g/l peptone water, 5.0 g/l tryptone, 0.4 g/l bile salts , 4.5 g/l yeast extract (Oxoid, Hampshire, UK), 0.0005 g/l $FeSO_4.7H_2O$, 4.5 g/l NaCl, 4.5 g/l KCl, 0.5 g/l $KH_2PO_4$, 1.25 g/l $MgSO_4.7H_2O$, 0.15 g/l $CaCl_2.6H_2O$, 1.5 g/l $NaHCO_3$ (Fischer scientific, Loughborough, UK), 0.8 g/l L-cysteine, 0.05 g/l hemin, 1.2 g/l glutamic acid, 1 ml Tween 80, 4 ml resazurin (0.025g/100ml, pH7) and 10 µl vitamin K. The gut model medium was prepared with compounds obtained from Sigma-Aldrich, Poole, UK unless otherwise stated.

### 2.1.1 Fermentation

A total of 6 gut model systems were set up with 3 systems representing each independent replicate for the two compounds to be tested. Each vessel was inoculated with 28.57 ml (V1), 33.33 ml (V2) and 37.50 ml (V3) of fresh 20 % (w/v) of faecal slurry from a healthy donor. The vessels were magnetically stirred and oxygen-free nitrogen was pumped overnight to allow the system to equilibrate before the medium pump was started. Oxygen-free nitrogen flow and pH were maintained throughout the whole experiment. After 8 turnovers (16 days) of the operating volume (300 ml in total) at a medium flow rate of 6.25 ml/h, SCFAs were analysed for 3 consecutive days to confirm the establishment of steady state. Then, after the first steady state (SS1) was achieved, the gut model system was fed with 13 g/l casamino acids or with 10 g/l L-cysteine until the second steady state was completed. Samples were taken for three consecutive days after confirmation of the equilibrium for

GABA analysis, SCFA analysis and DNA isolation followed by 16S amplification and sequencing to identify changes in the gut microbiota.

| | **Operating volume(ml)** | **Faecal inoculum (ml)** | **Media (ml)** | **Dilution rate ($h^{-1}$)** | **Retention time (h)** | **Minimum doubling time (h)** |
|---|---|---|---|---|---|---|
| V1 | 80 | 28.57 | 51.43 | 0.078 | 12.800 | 8.88 |
| V2 | 100 | 33.33 | 66.67 | 0.063 | 16.000 | 11.00 |
| V3 | 120 | 37.50 | 82.50 | 0.052 | 19.200 | 13.33 |
| TOTAL | 300 | | | | 48 | |

**Table 1**: Operating characteristics of the three-stage continuous culture fermentation system. With flow rate (ml $h^{-1}$) = total working volume/retention time, dilution rate ($h^{-1}$) = flow rate/total working volume, Minimum doubling time = 0.693/dilution rate and retention time (h) = the sum of the reciprocals of the dilution rate in each vessel.

### 2.2 GABA analysis

GABA analysis was performed according to Tsukatani et al.,( 2005) and Karatzas et al., (2010). One ml culture sample that was taken from the three stage continuous fermentation and was centrifuged at 11337 ×g for 10 min and the supernatants were stored in Eppendorf tubes and kept at - 80 $^{o}$C until it was required for analysis. GABAse master mix was prepared as such to contain 80 mM Tris (base) amino methane, 2 mM α-ketoglutarate,750 mM sodium sulphate , 10 mM dithiothreitol (DTT) , 1.4 mM β-nicotinamide adenine dinucleotide phosphate (NADP ) and 0.3 g/l of the enzyme GABASE from *Pseudomonas fluorescens* (Sigma Aldrich, UK) in sterile water. Ninety µl of the master mix was placed in each one of the wells of a 96-well plates and subsequently, 10 µl of the defrosted supernatant was added. In parallel, GABA standard solutions containing known concentrations of GABA (0, 2,4,6,8 and 10 mM) were prepared, added in wells containing the master mix and run alongside the

samples. The plate was then placed in a Sunrise plate reader (Sunrise, TECAN, Mannedorf, Switzerland) set at 37 $^{o}$C where absorbance at 340 nm was monitored every 2 min for 3 h and data was recorded using Magellan software (TECAN, Mannedorf, Switzerland) and analysed using Microsoft Excel.

**2.3 Short chain fatty acid (SCFA) analysis**

The SCFA in faecal samples was measured using Gas Chromatography- Mass Spectrometry (GC-MS) detection and the derivatisation method was modified from (Richardson et al., 1989).

Fifty µl of 0.1 M 2-ethylbutyric acid solution was added to 1 ml of faecal sample in Hungate tubes and vortexed. The mixture was acidified with 0.5 ml 30% HCl before adding 2 ml of diethyl ether. The mixture was properly vortexed and then centrifuged at 17000 ×g for 10 min. The diethyl ether (upper) layer of each sample was transferred to a labelled clean glass tube. Ether extract (0.4 ml) and 50 µl N-(tertbutyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA, Sigma-Aldrich, Poole, UK) were added into a 2 ml GC screw-cap vial. The mixtures were incubated at room temperature for 72 h to ensure that derivatisation was complete. Standard mixtures containing 5 mM, 2-ethyl butyrate for calibration were extracted and derivatised using the same steps as those for the faecal samples. Subsequently, the samples and standards were analysed using the GC.

Analysis was performed using an Agilent 6890/5975 GC-MS system (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5MS 30 m×0.25 mm column with a 0.25 µm coating of crosslinked 5% phenyl-methylpolysiloxane, (Hewlett Packard, UK) .The carrier gas (helium) had a flow rate of 1.7 ml min$^{-1}$ and head pressure of 113 kPa. The initial temperature was 63 °C and was elevated by 15 $^{o}$C min$^{-1}$ utill it reached 190 $^{o}$C and then holds for 3 min. The injector and detector temperatures were set at 275$^{o}$C. The test samples were

injected (1 µl) with a split ratio of 100:1. Quantification of the samples was achieved by calibration with acetic, propionic, butyric acid with concentration between 25 and 100 mM.

**2.4 Total DNA isolation for enumeration of microbial population using 16S sequencing**

DNA isolation was performed according to the protocol provided by the QIAamp Power Faecal DNA kit (Qiagen, Hilden, and Germany). Briefly, faecal samples obtained from each one of the vessels of the gut model (1 ml) were centrifuged (11337×g for 10 min) and the pellet was re-suspended in 750 µl of power bead solution (Qiagen). The mixture was added in the dry bead tube provided with the kit and 60 µl solution C1 (Qiagen) was also added followed by gentle vortexing. Subsequently, the tubes were placed in a water bath at 65 $^{o}$C for 10 min. The mixture was then vortexed for 10 min and centrifuged at 11337×g for 1 min. The supernatant was transferred to a 2 ml collection tube and 250 µl of solution C2 (QIAGEN) was added followed by brief vortexing. Then the mix was incubated at 4 $^{o}$C for 5 min and centrifuged at 11337×g for 1 min. Six hundred µl of the supernatant was carefully transferred into a 2 ml collection tube and 200 µl of C3 solution (QIAGEN) was added to the tube and then incubated at 4 $^{o}$C for another 5 min. Following a centrifugation of the mixture at 11337×g for 1 min, 750 µl of the supernatant was transferred to a clean 2 ml collection tube where 1200 µl of C4 solution (QIAGEN) was added to the supernatant and vortexed for 5 sec. The mixture was then carefully loaded into an MB spin column and centrifuged at 11337×g for 1 min. The flow through was discarded while 500 µl of solution C5 (Qiagen) was added to the spin column containing the DNA which was centrifuged at11337×g for 1 min. The spin column was then carefully placed in a clean 2 ml collection tube and 50 µl of solution C6 (QIAGEN) was added to elute the DNA followed by centrifugation at 11337×g for 1 min. Subsequently, the DNA concentration was assessed using a Nano drop spectrophotometer ND-1000 (Thermo-Fisher, UK) and the DNA was stored at -20 $^{o}$C for further amplification of 16S and sequencing. DNA concentration and purity was monitored

on 1% agarose gels. According to the concentration, DNA was diluted to 1 ng/μl using sterile water. This step was performed by Novogene, (Hong Kong).

### 2.6 16s bacterial profiling (Novogene, Hong Kong).

#### 2.6.1. 16S Gene Amplicon Generation

| Types | Region | Fragment length | Primer | Primer sequences ( 5'- 3 ) |
|---|---|---|---|---|
| Bacterial16S | V3-V4 | 466 bp | 341F | CCTAYGGGRBGCASCAG |
| | | | 806R | GGACTACNNGGGTATCTAAT |

**Table 2:** 16s gene amplicon generation. Showing the region, fragment length, primer with primer sequence.

The DNA sequence was amplified using the Polymerase chain reaction (PCR) and all PCR reactions were carried out in 30μL reactions with 15μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 0.2 μM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling was started with the initial denaturation at 98 ℃ for 1 min, followed by 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 60 s. Once all cycles were concluded samples were subjected to a final stage at 72 ℃ for further 5 min.

#### 2.6.2. PCR Products quantification and qualification

Equal volumes of 1X loading buffer was mixed (contained SYB green) with PCR products and electrophoresis was done on 2% agarose gel for detection. Samples with bright main strip between < 470bp was selected for sequencing.

### 2.6.3. PCR Products Mixing and Purification

PCR products was mixed in equidensity ratios. Then, mixture PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific).

### 2.6.4. Library preparation and sequencing analysis.

Standard bioinformatics analysis such as operational taxonomic units, alpha and Beta diversity, species distribution etc. was carried out. Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated.

# 3. Results

### 3.1 GABA production

Overall, the addition of casamino acids or L-cysteine resulted in a significant increase in GABA production at SS 2 compared to SS1 (Fig. 1). There was a significant increase in GABA (SS 2) of about 3-fold (from 0.8 to 2.2 mM) in the proximal (V1), approximately 3-fold (from 0.9 to 2.9 mM) in the transverse colon (V2) and a highly significant increase from 0 to 3.4 mM in the distal colon (V3) following the addition of casaminoacids (Fig. 1) Addition of L-cysteine also resulted in a highly significant increase in GABA of 7-fold (from 0.3 to 2.1 mM) in the transverse (V2) and 13-fold (from 0.1 to 1.3 mM) in the distal colon (V3) at SS 2 compared to SS1. There was also an increase of 2-fold (from 1.5 to 2.9 mM) in the proximal colon (V1) although it was not statistically significant. Casamino acids showed the highest concentration of GABA at the distal colon (pH 6.7- 6.9) while L-cysteine showed

the highest concentration of GABA at the proximal colon (pH 5.4 -5.6) which suggests casamino acids support GABA production with saccharolytic bacteria at while L-cysteine supports production of GABA with proteolytic bacteria.

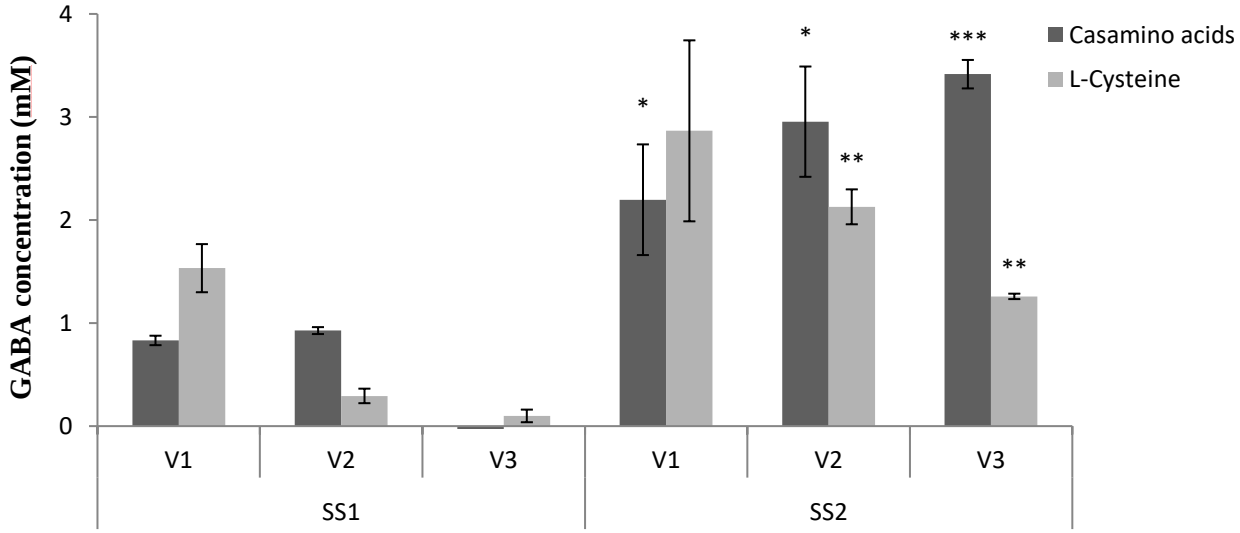



**Fig. 1.** Levels of GABA (mM) in the proximal (V1) transverse (V2) and distal (V3) colon following supplementation of the gut models with 13 g/l casamino acids or 10 g/l L-cysteine. Bars represent mean values from samples collected over three consecutive days from 3 gut models for each of the casaminoacids and L-cysteine fermentation. Error bars represent standard deviation of the mean values. Asterisk (*) denotes mean values at SS2 that were significantly different from SS1 ($P < 0.05$), double asterisk (**) denotes mean values at SS2 that were very significantly different from SS1 ($P < 0.01$) and triple asterisk (***) denotes mean values at SS2 that were highly significantly different from SS1 ($P < 0.001$).

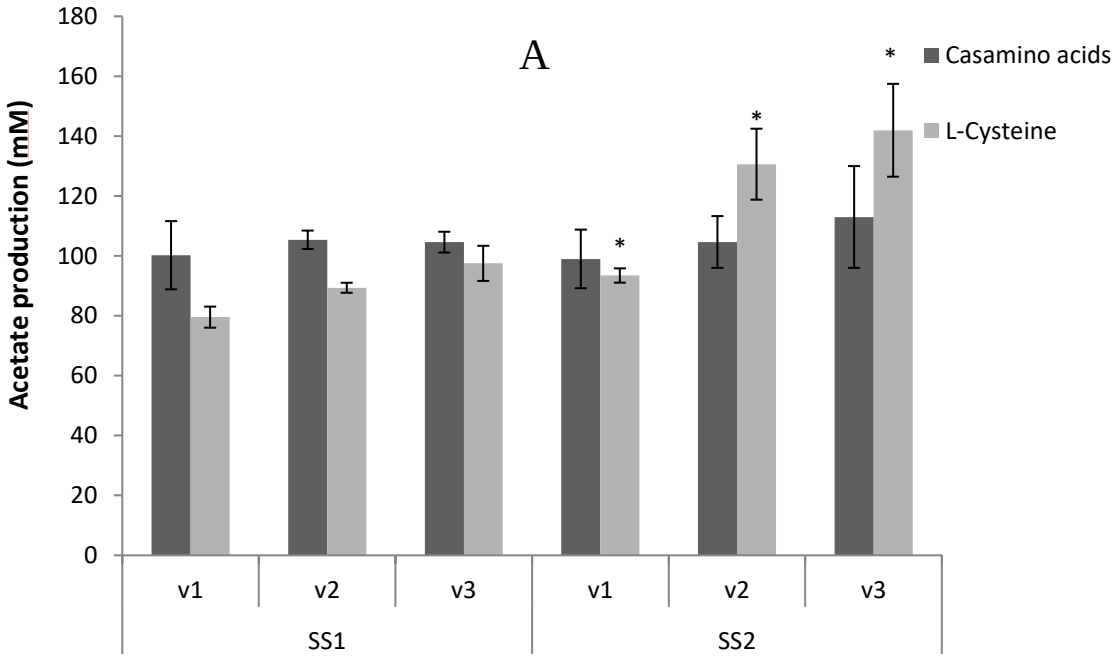
### 3.2 Short chain fatty acid production

SCFAs are produced by the fermentation of carbohydrates and protein although, carborhydrates where is the major contributor to the fermentation process. Our results (Fig. 2a) show that addition of L-cysteine resulted in a significant increase acetate levels by 2-fold (from 80 to 142 mM) in ($P < 0.05$) occurred in all vessels at SS 2 compared to SS1.

With the addition of 13 g/l casamino acids, there was a statistically significant increase of 2-fold (from 37 to 68 mM; $P < 0.05$) in butyrate levels at SS 2 compared to SS 1 in the

transverse (V2) and distal (V3) colon (Fig. 2B). Furthermore, addition of L-cysteine resulted in a statistically significant decrease of 2-fold (from 64 to 30 mM; $P < 0.05$) in butyrate levels in the proximal colon (V1) and a very significant decrease of 1-fold (from 71 to 57 mM; $P < 0.01$) in the distal colon (V3) in SS 2 compared to SS 1.

Addition of casamino acids and L-cysteine resulted in a statistically significant decrease in propionate production in all vessels at SS 2 compared to SS1 (Fig. 2C). The addition of 13 g/l casamino acids resulted in a highly significant decrease of approximately 2-fold (from 37 to 20 mM;$P < 0.001$) in the proximal colon (V1) and a significant decrease of 1-fold (from 46 to 32 mM; $P < 0.05$) in the transverse colon (V2). The addition of L-cysteine resulted in a significant decrease of 1-fold (from 49 to 38 mM; $P < 0.05$) in the transverse (V2) and of 1-fold (from 52 to 38 mM; $P < 0.05$) in the distal colon (V3).



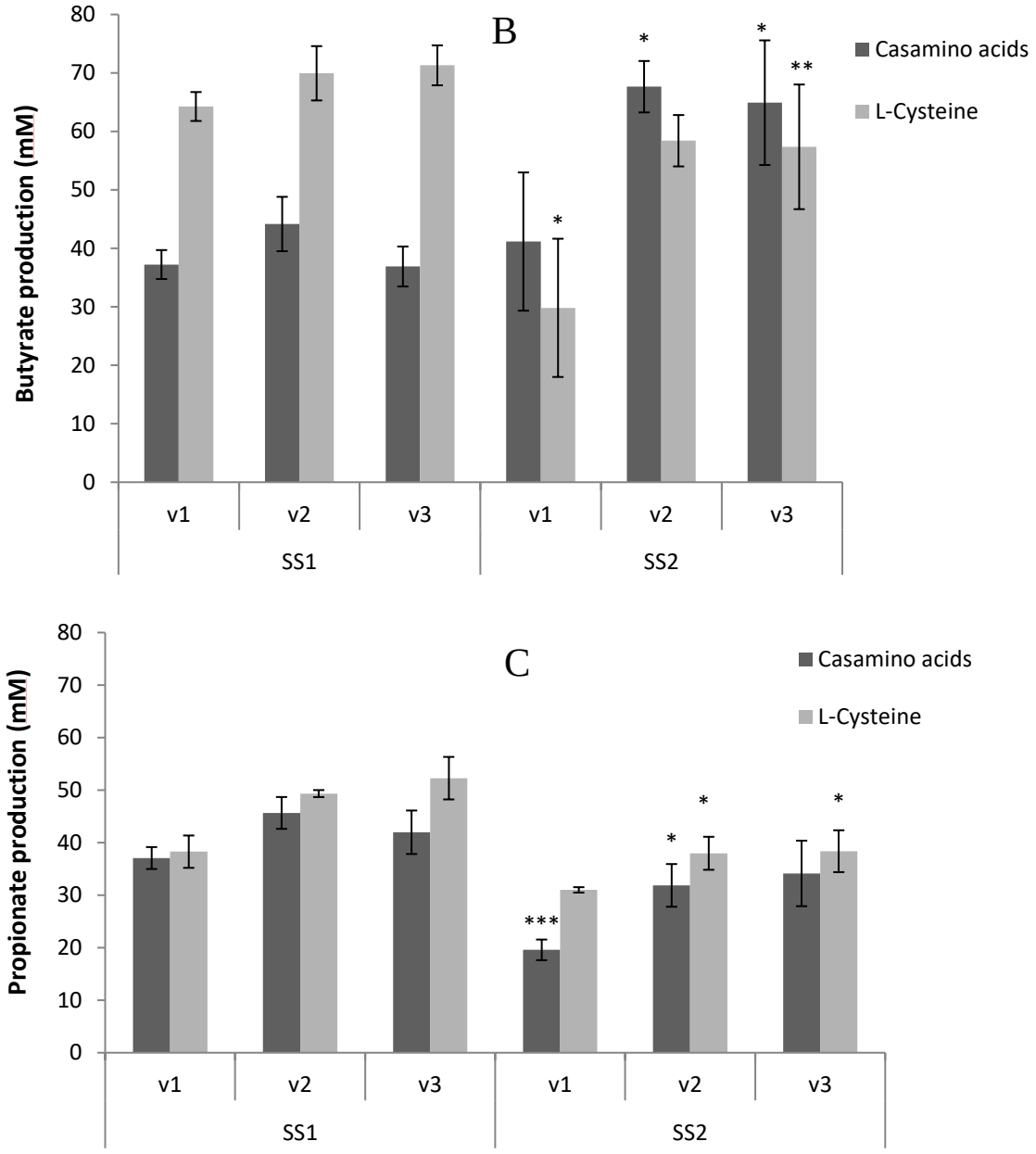



**Fig 2:** Concentrations of SCFAs acetate (A), butyrate (B) and propionate (C), in the proximal (V1) transverse (V2) and distal (V3) colon following supplementation of the gut models with 3.9 g day $^{-1}$ casamino acids or 0.9 g day $^{-1}$ L-cysteine. Bars are mean values over three consecutive days from 3 gut models for each of the casaminoacids or the L-cysteine fermentation. Error bars represent standard deviation of the mean values. Asterisk (*) denotes mean values that at SS2 were statistically significantly different from SS1 ($P < 0.05$), double asterisk (**) denotes mean values that at SS2 were very significantly different from SS1 ($P < 0.01$) and triple asterisk (***) denotes mean values that at SS2 were highly significantly different from SS1 ($P < 0.001$).

### 3. 3 16S sequencing

It is clear that addition of casamino acids or L-cysteine affected significantly the composition of the microbiota in the gut model (Fig 3).

The addition of casamino acids, resulted in a decrease in the numbers of bacteria from the *Dialister, Bacteroides* and *Prevotella* genera in all vessels and a decrease in *Bifidobacteria* in the proximal colon. There was also an increase in *Eschericha-Shigella* in the proximal (V1), transverse (V2) and distal colon (V3) and an increase of *Parabacteroides* in the transverse (V2) and distal (V3) colon. While there was an increase of over 25 % relative abundance in *Ezakiella* in the distal (V3) colon and an increase in *Eubacterium-rectale* in the proximal (V1) colon.

Addition of L-cysteine resulted in a significant decrease in *Dialister, Prevotella and Eubacterium-rectale* in all vessels and an increase in *Bifidobacterium* in the proximal (V1) colon and a decrease in the transverse (V2) and distal (V3) colon. There was also an increase in *Escherichia-Shigella* in all vessels and an increase in *Methanobrevibacter, Peptinophilus, Parabacteroides* and *Bacteroides* in the transverse (V2) and distal (V3) colon at SS2 compared to the baseline (SS1).

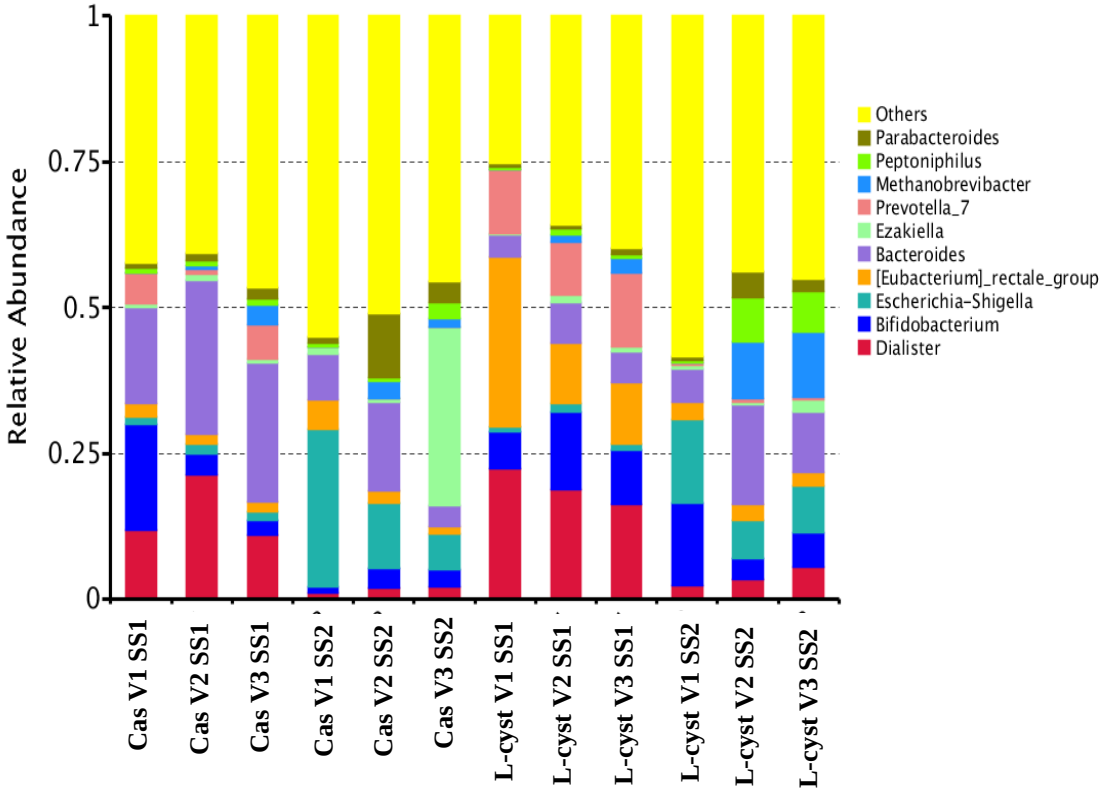



**Fig 3:** Operational taxonomic unit (OTU) analysis for the top 10 genera based on relative abundance from the 16S RNA sequencing of samples taken from vessels with the addition of casamino acids and L-cysteine. Cas V1 SS1, Cas V2 SS1 and Cas V3 SS1 represent microbiota in V1, V2 and V3 respectively at SS 1, while Cas V1 SS2, Cas V2 SS2, Cas V3 SS2 represent microbiota in V1, V2 and V3 respectively at SS 2, maintained with casamino acids supplementation. L-cyst V1 SS1, L-cyst V2 SS1, L-cyst V3 SS1 represent microbiota in V1,V2 and V3 respectively at SS 1 while L-cyst V1 SS2, L-cyst V2 SS2, L-cyst V3 SS2 represent microbiota in V1,V2 and V3 respectively at SS 2 maintained with L-cysteine supplementation.

### 3.4 SPSS (Pearson correlation) analysis correlation between bacterial profiling and GABA, acetate, butyrate and propionate levels.

With the use of SPSS software we were able to perform an analysis to identify through Pearson correlation (correlation co-efficient between +1 and -1) a possible correlation between bacterial genera or species and GABA, actetate or between SCFA levels. We found that several bacterial groups or genera showed a positive correlation while some had a negative correlation with GABA, acetate, butyrate and propionate levels (Table 3). For instance, non- classified /other groups, *Peptoniphilus, Eisenbergiella a*nd *Dialister* showed a positive correlation. Furthermore, *Ruminiclostridium, Eubacterium rectale, Enterococcus, Esherichia shigella* showed a negative correlation with GABA, acetate, propionate and butyrate respectively.

| Bacterial genera | GABA | Bacterial genera | Acetate | Bacterial genera | Butyrate | Bacterial genera | Propionate |
|---|---|---|---|---|---|---|---|
| Others | **0.625**** | *Peptoniphilus* | **0.524**** | *Eisenbergiella* | 0.398* | *Dialister* | **0.655**** |
| *Finegoldia* | 0.393* | *Slackia* | **0.497**** | *Eubacterium_rectale_group* | 0.396* | *Subdoligranulum* | **0.512**** |
| *Parabacteroides* | 0.386* | *Murdochiella* | **0.487**** | *Holdemanella* | 0.388* | *Parasutterella* | **0.505**** |
| *Terrisporobacter* | 0.353* | *Bilophila* | **0.479**** | *Subdoligranulum* | 0.357* | *Lachnospiraceae_FCS020_group* | **0.471**** |
| *Clostridium_sensu_stricto_15* | 0.352* | *Pyramidobacter* | **0.457**** | *Faecalicoccus* | 0.336* | *Ruminococcaceae_UCG_013* | **0.433**** |
| *Caproiciproducens* | 0.338* | *Clostridium_innocuum_group* | **0.454**** | *Bacteroides* | -0.330* | *Erysipelotrichaceae_UC_003* | 0.418* |
| *Anaerococcus* | 0.333* | *unidentified_Family_XIII* | **0.448**** | *Dielma* | -0.340* | Family*_XIII_AD3011_group* | 0.404* |
| *Ruminiclostridium_9* | -0.342* | *Lachnoclostridium* | 0.416* | *Butyricimonas* | -0.343* | *Akkermansia* | 0.392* |
| | | *Anaerofustis* | 0.406* | *Others* | -0.368* | *Desulfovibrio* | 0.389* |
| | | *Mogibacterium* | 0.377* | *Enterococcus* | -0.425** | *Pseudomonas* | 0.382* |
| | | *Eisenbergiella* | 0.369* | | | *Odoribacter* | 0.349* |
| | | *Catabacter* | 0.356* | | | *Blautia* | 0.348* |
| | | *Ruminiclostridium* | 0.345* | | | *Clostridium_sensu_stricto_1* | -0.337* |
| | | *Pseudobutyrivibrio* | 0.340* | | | *Enterorhabdus* | -0.350* |
| | | *Rhodococcus* | 0.340* | | | *Escherichia_Shigella* | -0.402* |
| | | *Lactonifactor* | 0.336* | | | | |
| | | *Eubacterium_rectale_group* | **-0.473**** | | | | |

**Table 3:** Pearson correlation between bacterial genera and GABA, acetate, butyrate or propionate. : (A), (B), (C) and (D) is the correlation between bacterial genera profile and GABA, acetate, butyrate and propionate levels respectively throughout the whole experiment and in all

three vessels. Asterisk (*) indicates significant correlation (P<0.05; 2-tailed) and double asterisk (**) indicates very significant correlation (P<0.01; 2-tailed) and N which is the sample size = 36.

# 4. Discussion

The human gut is colonised by several trillions of bacteria which are important for health as they contribute to the gut metabolism by breaking down complex polysaccharides that are ingested by the host. It has been suggested that gut microbiota has the ability to communicate with the brain and modulate mood and behaviour by producing neuroactive metabolites which can affect the central nervous system (CNS) (Foster and McVey Neufeld, 2013). There is an emerging literature showing a two-way interaction between the brain and gut which has been implicated in several health problems such as autism spectrum disorders (ASD), anxiety, depression, changes in mood and behaviour and also diseases such as irritable bowel syndrome (IBS) (Park et al., 2013, Cryan and Dinan, 2012, Mayer et al.). For example, alterations in the intestinal microbiota are implicated in ASD and several complex behaviours and also contribute to the brain function and development in mice (Bercik et al., 2011, Cryan and Dinan, 2012, Neufeld et al., 2011) and in humans (Tillisch et al., 2013). GABA has also been reported to slow down the proliferation of cancer cells as it is considered to help in suppressing tumours (Watanabe et al., 2006, Schuller et al., 2008). Likewise, existing research has shown the blood-pressure lowering effect of GABA on spontaneously or mildly hypertensive rates which could potentially be helpful in managing various cardiovascular diseases (Aoki et al., 2003, Inoue et al., 2003). The gut microbiota is known to produce various neuroactive metabolites and these might result in the effect on the brain. It has been shown that the vagus nerve plays an important role in some of these effects, while it is not known if the metabolites activate the vagus nerve or the bacteria themselves activate the vagus nerve through a different route (Cryan and Dinan, 2012, Dinan and Cryan, 2017). Intestinal microbes have shown to improve mood, reduce anxiety in chronic fatigue patients and also it has been shown to reduce reactivity to sad mood by healthy subjects (Logan and

Katzman, 2005, Steenbergen et al., 2015) and as such research on this subject is highly important for health.

Gut microbiota respond differently to various environmental conditions which affect their growth and balance between various species or genera. This is clear as changes in available substrates affect metabolism and this can favour or not specific members of the gut microbiota.

Due to the various physiological and psychological health benefits of GABA which was stated above, scientists over the years have focused on increasing GABA levels in the gut mainly through GABA enriched foods of which most are fermented. These foods have have been observed to show mixed results with regards to their effects on health (Diana et al., 2014, Boonstra et al., 2015). Nutrients such as sugars, amino acids, dietary fibres etc. when ingested by the host, may be absorbed and converted into metabolites such as GABA by the gut microbiota and these metabolites may act and regulate various functions in the host (Hemarajata and Versalovic, 2012). On the other hand, these metabolites might also be catabolised by other microbes which in their turn might produce other metabolites. Therefore, an excess in a specific compound can be created in the gut when its catabolism by the gut microbiota is slower than its production.

Regarding GABA, up to now the work has focused on levels of GABA in foods with the idea that high levels of GABA in foods such as various fermented or GABA-enriched foods, could provide high levels of GABA to the gut guaranteeing an excess that can elicit its health effects. However, the gut microbiota have the capability of producing GABA and little work has been done on how they could be utilised as a tool to provide this excess of GABA in the gut.

The present study is one of the first trying to identify environmental conditions, focusing mainly on compounds that are present in food that could increase GABA levels in the gut upon ingestion. Therefore, increasing the levels of these nutrients in the gut could possibly increase the ability of the gut microbiota to produce higher levels of GABA. We found an increase in the GABA levels in our previous experiment on single –stage batch culture fermentation supplemented with casamino acids and L-cysteine. Although, single–stage batch fermentation system is quite limited, therefore we had to further study the effects of these amino acids on GABA levels using the gut model which is a better representation of the human large intestine. Our results here show that supplementation with casamino acids or L-cysteine resulted in a significant increase in GABA levels throughout the gut model in all 3 vessels simulating the proximal, transverse and distal colon in SS 2 compared to the baseline (SS1). With the addition of casamino acids (Fig. 1), that there was a highly significant increase in GABA in the distal (V3) colon and this could possibly be as a result of all the carbohydrates been depleted in the distal colon while the casamino acids provides nitrogen or protein source for metabolic activity by the proteolytic bacteria in the distal colon thus boosting the production of GABA in the distal colon. Whereas with L-cysteine, there was an increase in levels of GABA in the proximal (V1) colon which suggests that L-cysteine supports GABA production in saccharolytic bacteria which are abundant in the proximal colon which has a high carbohydrate availability , it could also be suggested that the low pH in the proximal colon as well as the addition of L-cysteine which has the ability to increase the hydrogen production in the system was able to activate the GAD system to produce more GABA in the proximal colon .

The gut microbiota is involved in the utilization of several peptides and amino acids which can serve as precursors for the metabolic end products (GABA and SCFAs) produced by the intestinal microbiota as the gut microbiota utilizes amino acids for the synthesis of metabolic

end products (Lin et al., 2008). In this work we found that the addition of L-cysteine resulted in a significant increase in the levels of acetate throughout the gut model in all vessels in SS 2 compared to SS 1. This could possibly be as a result of an increase in the production of hydrogen which in turn lowers the pH of the system causing a shift in metabolic pathways to increase the acetate and butyrate concentration in anaerobic mixed cultures (Yuan et al., 2008). However, in our gut models (fig 2b), in contrast to what described by Yuan et al. (2008) we found a significant decrease in butyrate and propionate production following the addition of L-cysteine. It has been reported that L-cysteine increases hydrogen production and yield, and hydrogen is the main substrate responsible for methanogenesis in the gut, therefore increased digestibility of fibrous materials may result in an increased methane production during microbial fermentation (Takahashi et al., 2000). *Methanobevibacter* improves the efficiency of polysaccharides in animal gut fermentation by preventing the build-up of hydrogen and other metabolic pathways by using up the methane produced at the end of the fermentation process (Armougom et al., 2009, Samuel et al., 2007). It has been reported by Samuel et al., (2007) that the survival of *Methanobrevibacter* was prolonged in the distal colon which we also found (Fig. 3) and this could possibly be linked to the ability of *Methanobrevibacter* to consume other fermentation products deriving from saccarolytic bacteria or as a result of effective competition for nitrogenous nutrients in the colon. Also, the bacterial hydrogen metabolism influences SCFA formation, therefore an increase in bacteria consuming hydrogen affects the partial pressure in the gut and hence, affects the formation of SCFAs as this influences the total balance of fermentation products formed (Louis and Flint, 2017, Wolf et al., 2016)

In recent years, there has been a noted progress in understanding the metabolism of SCFA in the human gut. Therefore, the production of SCFA is a major physiological process, which is supported by the gut microbiota (Macfarlane and Macfarlane, 2007). The OTU analysis (Fig

3) showed a reduction/disappearance of *Prevotella* and likewise a reduction in *Dialister* in the proximal, transverse and distal colon after feeding with both casamino acids and L-cysteine and this could possibly explain the reduction in the production of propionate (Fig. 2c) as they are both known to produce propionic acid as a metabolic end product (Macfarlane and Macfarlane, 2012, Jumas-Bilak et al., 2005). In addition, there was an increase *Escherichia – Shigella* (fig 3) with the addition of both casamino acids and L-cysteine at SS 2. *Escherichia – Shigella* is known to possess the glutamate decarboxylase (GAD) enzyme that is one of the most potent acid resistance systems in bacteria and the main bacterial system exporting GABA and would be mainly responsible for the increase in GABA levels in the gut (Waterman and Small, 2003, Feehily and Karatzas, 2013). Therefore the increase in *Escherichia – Shigella* could be linked to the significant increase in GABA production (Fig. 1) (Cotter et al., 2001).

Finally, from the Pearson's correlation carried out on the data collected from 16S sequencing using SPSS at genus level (Table 3), we found a highly significant correlation between *Dialister, Subdoligranulum, Parasutterella* and propionate production with a Pearson's correlation coefficient (R) of 0.655**, 0.512** and 0.505** respectively. It has been reported that the metabolic end products of *Dialister* is acetate, lactate and propionate and that *Dialister* is able to decarboxylate succinate present in the growth medium to produce propionate (Morotomi et al., 2008, Jumas-Bilak et al., 2005). Furthermore, trace levels of propionate have been found as a metabolic end product of *Parasutterella* in human (Nagai et al., 2009). In addition, *Peptoniphilus* and *Slackia* showed a positive correlation to acetate production and it was reported that major amounts of acetic acid was found in the metabolic end products of *Peptoniphilus* while moderate amount of butyrate was found and trace amount of propionate was detected (Rooney et al., 2011, Patel et al., 2016) likewise acetate, lactate and formate were also reported as the metabolic end products of *Slackia* (Nagai et al.,

2010) which supports our findings from this study (figure 3) that these bacteria genera have a positive significant correlation with the SCFAs produced.

Also, there was a positive correlation between *Eisenbergiella* to butyrate and acetate production and a positive correlation between *Eubacterium_rectale*_group to butyrate production (table 3). These results were also reported in different studies conducted by previous researchers who reported similar results from the metabolic end product of fermentation from isolated strains of these bacterial genera (Jumas-Bilak et al., 2005, Nagai et al., 2009, Nagai et al., 2010, Amir et al., 2014, Macfarlane and Macfarlane, 2012, Louis and Flint, 2009) .

# 5. Conclusion

In Conclusion, given the link between the gut microbiota and health, thereby modulating the gut microbiota through diet could be a possible strategy for reducing these health risks. Amino acids can be metabolised for the synthesis of bacterial cell components or catabolised via various pathways and the levels of these amino acids could positively or negatively affect the host. As it was observed from this study that the addition of these amino acids increased GABA and SCFA levels in the gut which could positively affect the gut health and in turn affect the host health. Therefore, modulating dietary protein or amino acid intake may be essential for intestinal microbiota and their metabolic pathways thereby potentially affecting the host metabolism.

# 6. Acknowledgement

The authors would like to thank all colleagues at the Microbiology research team, Food and Nutritional Sciences, University of Reading, Centre for Integrative Neurosciences and Neurodynamics (CINN) for the grant given towards the sequencing, Society for Applied Microbiology (SFAM) for the studentship grant given and Novogene, Hong-kong for 16S bacterial profiling including the library preparation, sequencing and bioinformatics analysis.

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# Chapter 6

# General discussion and future perspectives

## 6.1 General discussion

The gut is an essential organ in the human body that is responsible for digestion, absorption and metabolism of dietary nutrients and substrates, it contributes to approximately 12 % of the protein synthesis in the entire body and it is an essential route in which food proteins, natural toxins, pathogens and commensal gut flora enters the body (Wang et al., 2009). The gut microbiota plays a key role in activating the immune system and central nervous system where existing studies have shown the development of the brain system is dependent on the gut microbiota (Evrensel and Ceylan, 2015). As such, the gut microbiota is now considered as an endocrine organ that can affect the health of the host (Baquero and Nombela, 2012).

The gut microbiota colonise the gut and are able to produce and deliver neuroactive substances /metabolites such as γ-aminobutyric Acid (GABA), which are beneficial to the health of the host.

GABA is a major inhibitory neurotransmitter of the central nervous system, it is responsible for regulating many physiological and psychological processes and a disturbance in the GABA system in human has an implication on inducing anxiety and depression (Cryan and Dinan, 2012). GABA is also involved in regulation of cardiovascular conditions such as blood pressure; also, it has further potential health benefits such as regulating the secretion of growth hormones and having anti-proliferative effects on colon cancer cells (Joseph et al., 2002, Mody et al., 1994, Volpi et al., 1997). Therefore, with the major health benefits of GABA, this study is aimed at rapid identification of GABA producers using the colorimetric screening method,

selecting for GABA producers for their probiotic properties as well as enhancing the production of GABA in the gut through *in–vitro* studies by supplementing with various amino acids and peptides. Amino acids are important substrates for the synthesis of proteins and other nitrogenous compounds; they are also major regulators of refluxes through various metabolic pathways (Wang et al., 2009, Jobgen et al., 2006).

Previous studies have shown quantification of GABA using chromatography-based techniques such as high performance liquid chromatography (HPLC) which are laborious and expensive to run especially when identifying GABA production in thousands of isolates. In **chapter 2**, the colorimetric pre-screening method was developed to be able to identify and screen out major GABA producers from non-GABA producers between isolates grown in different environments before using the chromatography-based techniques. The GABA producers when cultured on a colorimetric agar plate containing pH indicators methyl red and methylene blue showed a bright green colouration. This colouration occurs due to the reduction in internal pH of the cells which in turn activates the glutamate decarboxylase (GAD) system (a mechanism of acid resistance in bacterial cells) and hence induces the production of GABA. This is the first time a pre-screening method has been developed for identifying GABA producers and it is a simpler, cost effective method, fast and less laborious pre-screening method of identifying GABA producers from a population of isolates in different environments.

The isolated lactic acid bacteria (LAB) strains from the gut were further tested in **chapter 3** for their probiotic potential as LAB are majorly used as starter cultures and probiotics in food and pharmaceutical processes as they are generally regarded as safe (GRAS) (Collado et al., 2007, Tannock, 1997). For a LAB, strain to be classified as a probiotic or starter culture it has to be tested for its safety and its ability to survive various environmental conditions found in the gut and confer a health benefit to the host (Morelli, 2007, Hill et al., 2014). Interestingly, looking into the potential health benefits of GABA, the thought of carrying out various tests on the

GABA producing strains for their probiotic properties was not far-fetched. The results showed that the GABA producing strains, which were majorly *Lactobacillus plantarum*, were more acid resistant and strong biofilm formers, which are properties that are advantageous in food production processes. The *L. plantarum* strains also had a low ability to acidify milk, which could possibly have an effect on the storage and shelf life of the product. Two of the GABA producing *L. plantarum* strains were selected for batch culture fermentation to determine their effect on GABA production in the gut. There was a slight increase in GABA production at 12 h and 48 h although, the increase was not significant therefore future work is recommended to determine if the increase in GABA is because of inoculating with GABA producers.

Approximately 3-12 g of dietary proteins and peptides which are nitrogen sources reaches the large intestine daily and there has been a growing interest in the physiological and nutritional effects of amino acids on health (Smith and Macfarlane, 1996). Though existing data shows that supplementing diet with amino acids enhances gene expression, regulates neurological development and function, and also stimulates protein synthesis (Wu, 2013). Likewise, various studies have been carried out to show the health benefits of GABA production as well as the effect of individual bacterial strains on GABA production (Huang et al., 2007, Cho et al., 2007, Choi et al., 2006, Barrett et al., 2012). There is no evidence of the effects on GABA production in the gut when supplementing with amino acid mixtures and peptides. This is the first time the effect of amino acids and peptides such as casamino acids, tryptone, peptone, L-cycsteine, tryptophan and yeast extract on modulating GABA and SCFA production have been studied. In both **chapter 4** and **chapter 5**, fermentation metabolites such as GABA and SCFA (acetate, butyrate and propionate) in *in vitro* models were monitored. The batch culture fermentation simulates the distal colon in the large intestine while the gut model simulates the proximal, transverse and distal colon. In **chapter 4,** the amino acid mixtures and peptides added increased the production of GABA, there was also a noticeable difference in results from the pH-

controlled, and the non-pH-controlled which suggests that environmental conditions affect the metabolism and bacterial growth in the gut. Interestingly, with both fermentation processes in **chapters 4 & 5** the amino acid mixtures and peptides increased the GABA production in the gut which is what is expected but there was a reduction in beneficial bacteria for instance *Bifidobacterium* and an increase in pathogenic bacteria such as *Escherichia-Shigella* and *Lachnoclostridium.* Therefore, supplementing with amino acids not only increased the production of metabolites that are beneficial to the human health but also the increases the growth of *Escherichia* and *Clostridia* group. This possible detrimental effect of amino acids and peptides could be significant to population that are set on specific diet such as people on a high protein as well as body builders who take protein supplements to increase muscle gain. As the proteins are broken down into amino acids which could both be beneficial in terms of producing metabolites as well as being detrimental by increasing the growth of some pathogenic bacteria.

In conclusion, we were able to isolate GABA producing bacteria that could serve as probiotic in functional food processes that could be beneficial to host health. Furthermore, we can infer that specific amino acid or mixture of amino acid and peptides in addition to dietary fibres and prebiotics are beneficial to gut and in turn beneficial to the host. Therefore, a deeper and clearer understanding of the potency of such supplements to maintain gut microbiota has the ability to contribute essential therapeutic tools in human metabolic health.

### 6.2 Limitation and future perspectives

In **chapter 2**, although the colorimetric screening test was carried out using MRS, Wilkins Charlgren and nutrient agar. It could be helpful to broaden the use of this technique to other growth media that could be beneficial for use on a larger and wider scale.

Likewise, in **chapter 3,** LAB strains isolated were assessed for their safety to use as probiotics but their technological features in food processing need to be further developed and their effects on storage and shelf life of functional or fermented food products need to be assessed.

In **chapter 4 & 5,** the application of *in vitro* models to determine the mechanisms and effects of amino acid on colon was studied. Batch culture fermentation was used to test different substrates over a short period of time, which allows us to be able to choose the most effective and relevant for use in the continuous stage model **(Chapter 5)**. Casamino acid and L-cysteine were selected as casamino acids contains all essential amino acids except tryptophan and L-cysteine is a non-essential amino acid and it would be interesting to see their different metabolic pattern in the gut system. In **chapter 5**, the three-stage continuous culture fermentation system was used simulating the three distinct region of the human colon. Protein fermentation occurs in the distal colon and it is difficult to study with non – invasive human trials. In addition, metabolites accumulate towards the end of fermentation in batch culture systems while metabolites accumulate from the proximal to the distal end of the continuous culture model but the absorption ability could not be researched, as the *in vitro* models used do not have the capacity for this function. In general, the short falls of *in vitro* models is that they lack host interactions like immune responses, absorption and secretion while they are useful in simulating the gut to promote early research screening (Macfarlane and Macfarlane, 2007). Furthermore, the breakdown of protein and amino acid metabolism results in the formation of potentially toxic metabolites such as ammonia, indole, amine and p-cresol (Macfarlane and

Macfarlane, 1997). The formation of these potentially toxic metabolites were not included in this research work but it will be helpful to have the data on these metabolites in future work.

In *in-vitro* studies, bacterial and metabolic responses differ in fermentation with different donors. The selection criteria for the donors used was based on the use of drugs and antibiotics and also disease however, there are other factors such as stress, lifestyle and dietary habits that could influence the gut microbiota and its metabolic function (Nicholson et al., 2012). This could affect the results from the fermentation process such as the wide margin in standard deviation. Therefore, lifestyle and dietary habits should be included in selection for conduction in vitro studies.

### 6.3 Future Work

This research work has explored methodologies and analyses that have provided good and strong background to GABA production in lactic acid bacteria as well as the modulation of GABA production in the gut using dietary interventions.

The research presented in this thesis seems to have raised more questions along the line which could not be explored due to time constraints and there are other lines of research arising which could be pursued in the future.

1. Following the isolation GABA producing LAB strains and the test for probiotic potential, the strains could be introduced into food as starter culture in fermented food to see if there will be GABA production as well as test their effects on storage and shelf life of the food product. Also, it would be interesting to feed this functional fermented foods into the gut models to see if there will be any effect on GABA levels in the gut, the gut microbiota and the host.

2. Apart from increasing GABA and SCFA concentration in the gut, amino acids and peptides could also increase the levels of some toxic metabolites in the gut such as p-cresol, amine, ammonia and indole therefore the first step is to see the production of these toxic compounds gut .
3. Following this study on modulation GABA production in the gut by supplementing with amino acids in the gut model simulation the human colon, it will be interesting to translate this experiments into animal models to see the effect of the dietary interventions on the animal models and then possible in human intervention trials.

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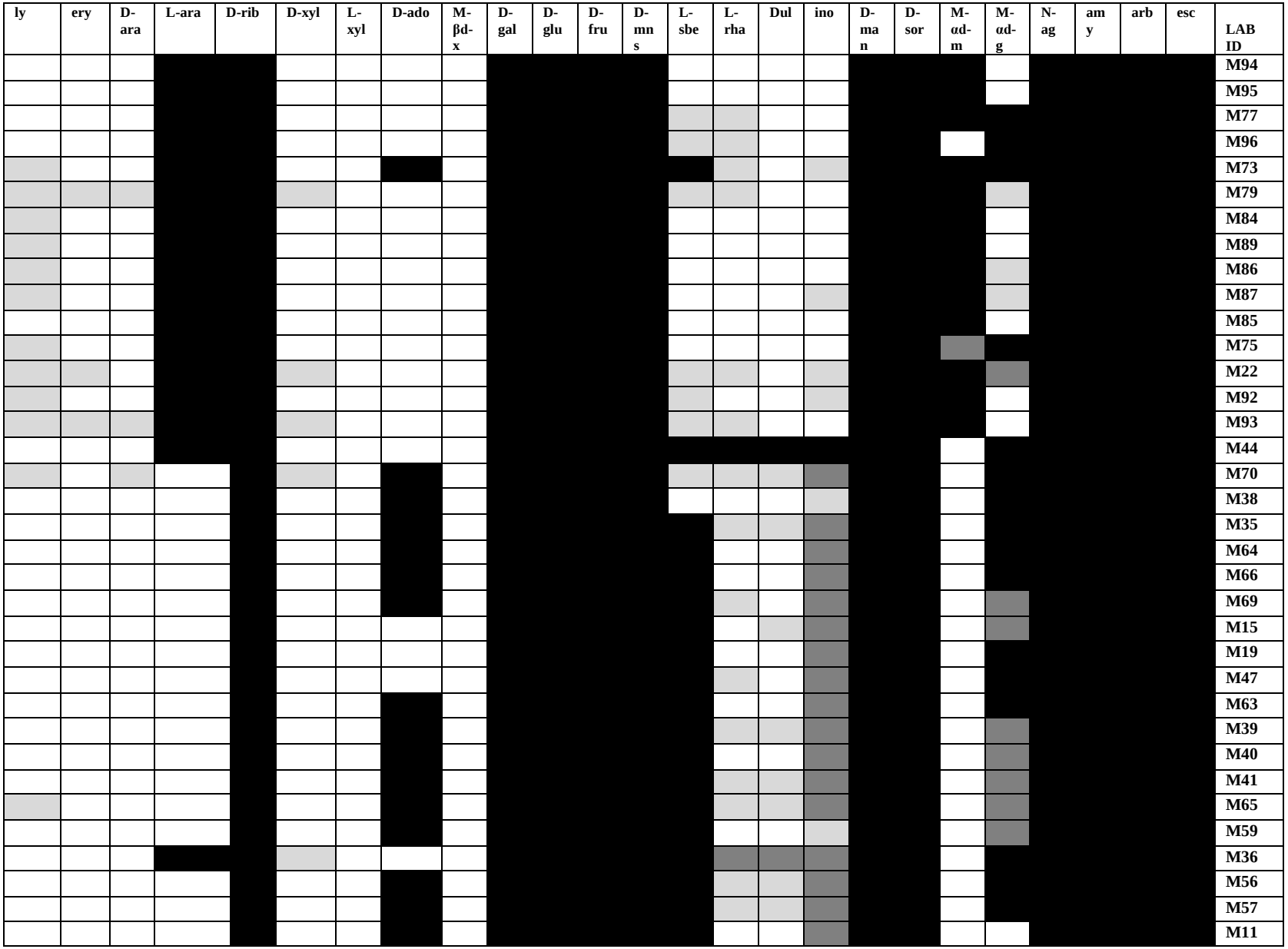
# APPENDICES

## 3.1 Biochemical profile of faecal isolates on the *apiweb*$^{TM}$ identification software



| ly | ery | D-ara | L-ara | D-rib | D-xyl | L-xyl | D-ado | M-βd-x | D-gal | D-glu | D-fru | D-mns | L-sbe | L-rha | Dul | ino | D-man | D-sor | M-αd-m | M-αd-g | N-ag | amy | arb | esc | LAB ID |
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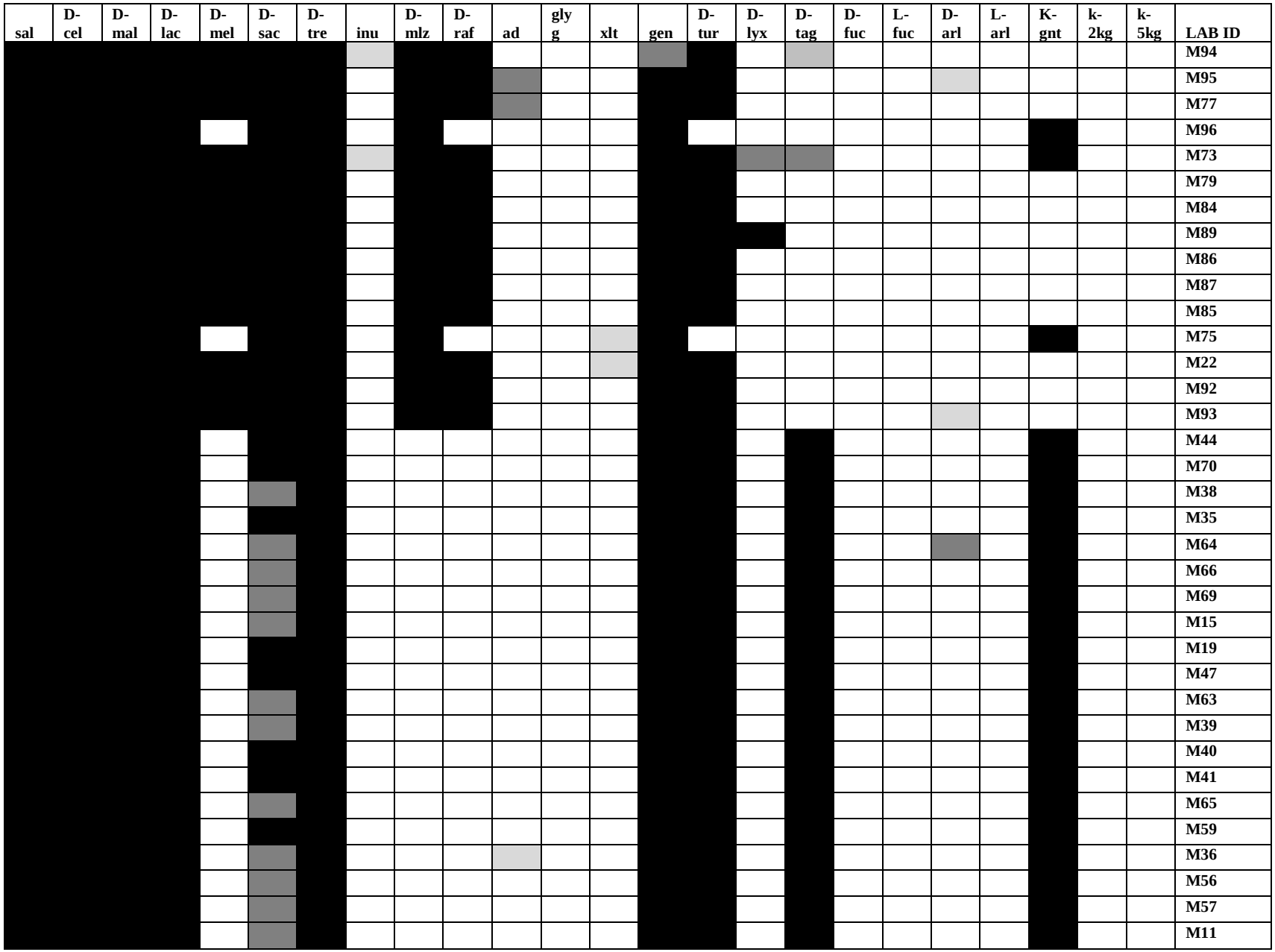

| sal | D-cel | D-mal | D-lac | D-mel | D-sac | D-tre | inu | D-mlz | D-raf | ad | gly g | xlt | gen | D-tur | D-lyx | D-tag | D-fuc | L-fuc | D-arl | L-arl | K-gnt | k-2kg | k-5kg | LAB ID |
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**KEY:** **Gly- Glycerol** **ery- ERYthritol** **D-ara- D-ARAbinose** **L-ara- L-ARAbinose** **Positive**

**D-rib- D-RIBose** **D-xyl- D-XYLose** **L-xyl- L-XYLose** **D-ado- D-ADOnitol** **Negative**

**M-βd-x- Methyl-βD-Xylopyranoside** **D-gal- D-GALactose** **D -glu- D-GLUcose** **Weak positive**

**D-fru- D-FRUcose** **D-mns- D-MaNnose** **L-sbe- L-SorBosE** **L-rha –L-RHAmnose** **Very weak positive**

**Dul- DULcitol** **ino- INOsitol** **D-man- D-MANnitol** **D-sor- D-SORbitol**

**M-αd-m- Methyl- αD-Mannopyranoside** **M-αd-g- Methyl- αD-glucosamine**

**N-ag- N-AcetylGlucosamine** **amy- AMYgdain** **arb- ARButin** **esc- ESCulin ferric citrate**

**sal- SALicin** **D-cel- D-CELiobiose** **D-mal- D-MALtose** **D-lac-D-LACtose**

**D-mel-D-MELibiose** **D-sac- D-SACcharose** **D-tre- D-TREhalose** **inu- INUlin** **D-mlz**

**D-raf- D-RAFfinose** **ad-AmiDon (Starch)** **glyg-GLYcogen** **xlt-XyLiTol**

**Gen- GENtiobiose** **D-tur-D-TURanose** **D-lyx- D-LYXose** **D-tag- D-TAGatose**

**D-fuc- D-FUCose** **L-fuc- L-FUCose** **D-arl- D-ARabitol** **L-arl- L-ARabitoL**

**K-gnt- Potassium GlucoNaTE** **k-2kg- Potassium 2-keto Gluconate** **k-5kg Potassium 5-keto Gluconate**

**KEY:**

| | | | |
|---|---|---|---|
| **M94 - *L. plantarum*** | **M85 - *L. plantarum*** | **M66 - *L. paracasei*** | **M59 - *L. paracasei*** |
| **M95 - *L. plantarum*** | **M75 - *L. plantarum*** | **M69 - *L. paracasei*** | **M36 - *L. rhamnosus*** |
| **M77 - *L. plantarum*** | **M22 - *L. plantarum*** | **M15 - *L. paracasei*** | **M56 - *L. paracasei*** |
| **M96 - *L. plantarum*** | **M92 - *L. plantarum*** | **M19 - *L. paracasei*** | **M57 - *L. paracasei*** |
| **M73 - *L. plantarum*** | **M93 - *L. plantarum*** | **M47 - *L. paracasei*** | **M11 - *L. paracasei*** |
| **M79 - *L. plantarum*** | **M44 - *L. rhamnosus*** | **M63 - *L. paracasei*** | |
| **M84 - *L. plantarum*** | **M70 - *L. paracasei*** | **M39 - *L. paracasei*** | |
| **M89 - *L. plantarum*** | **M38 - *L. paracasei*** | **M40 - *L. paracasei*** | |
| **M86 - *L. plantarum*** | **M35 - *L. paracasei*** | **M41 - *L. paracasei*** | |
| **M87 - *L. plantarum*** | **M64 - *L. paracasei*** | **M65 - *L. paracasei*** | |

## 4.1 SPSS (Pearson correlation) analysis correlating bacterial profiling at specie level with GABA, Acetate, butyrate and propionate production for pH controlled fermentation.

| Pearsons Correlations (N= 40) | GABA | Pearsons Correlations (N= 40) | Acetate | Pearsons Correlations (N= 40) | Butyrate | Pearsons Correlations (N= 40) | Propionate |
|---|---|---|---|---|---|---|---|
| *Dorea_longicatena* | 0.517** | *Butyrate* | 0.670** | *Propionate* | 0.680** | *Butyrate* | 0.680** |
| *Alistipes_sp._N15.MGS_157* | 0.421** | *Propionate* | 0.549** | *Acetate* | 0.670** | *Acetate* | 0.549** |
| *Peptoniphilus_sp._BV3AC2* | 0.405** | *Ruminococcaceae_bacterium_GD6* | 0.479** | *Ruminococcaceae_bacterium_GD6* | 0.515** | *Clostridium_sp._NML_04A032* | 0.485** |
| *Phascolarctobacterium_faecium* | 0.385* | *Anaerotruncus_sp._MT15* | 0.376* | *Anaerotruncus_sp._MT15* | 0.407** | *Alistipes_finegoldii* | 0.453** |
| *Pyramidobacter_piscolens* | 0.361* | *Methanobrevibacter_smithii* | 0.362* | *Others* | 0.370* | *Anaerotruncus_sp._MT15* | 0.415** |
| *Slackia_isoflavoniconvertens* | 0.348* | *Clostridium_paraputrificum* | 0.361* | *Alistipes_putredinis* | 0.363* | *Clostridium_sp._GD3* | 0.410** |
| *Alistipes_putredinis* | 0.332* | *Clostridium_sp._GD3* | 0.350* | *Dorea_formicigenerans* | 0.350* | *Dielma_fastidiosa* | 0.395* |
| *Methanobrevibacter_smithii* | 0.331* | *Butyricimonas_virosa* | 0.344* | *Alistipes_finegoldii* | 0.322* | *Ruminococcaceae_bacterium_GD6* | 0.372* |
| *Dorea_formicigenerans* | 0.316* | *Clostridium_butyricum* | 0.336* | | | *Streptococcus_gallolyticus_subsp._macedonicus* | 0.364* |
| *Enterococcus_faecalis* | -0.337* | *Parasutterella_excrementihominis* | 0.323* | | | *Sutterella_wadsworthensis* | 0.363* |
| *Hafnia_alvei* | -0.356* | *Parabacteroides_merdae* | -0.332* | | | *Collinsella_aerofaciens* | 0.325* |
| *Bacteroides_salyersiae* | -0.384* | *Anaerostipes_hadrus* | -0.375* | | | *Anaerostipes_hadrus* | -0.324* |
| | | *Haemophilus_parainfluenzae* | -0.376* | | | *Blautia_obeum* | -0.333* |

## 4.1 SPSS (Pearson correlation) analysis correlating bacterial profiling at specie level with GABA, Acetate, butyrate and propionate production for non-pH controlled fermentation.

| Pearsons Correlations (N=50) | GABA | Pearsons Correlations (N=50) | Acetate | Pearsons Correlations (N=50) | Butyrate | Pearsons Correlations (N=50) | Propionate |
|---|---|---|---|---|---|---|---|
| Bacteroides_thetaiotaomicron | 0.679** | Propionate | 0.852** | Propionate | 0.836** | Acetate | 0.852** |
| Dialister_pneumosintes | 0.669** | Butyrate | 0.762** | Acetate | 0.762** | Butyrate | 0.836** |
| Peptoniphilus_sp._BV3AC2 | 0.531** | Blautia_producta | 0.677** | Dorea_formicigenerans | 0.546** | Dorea_formicigenerans | 0.642** |
| Megasphaera_elsdenii | 0.519** | Dorea_formicigenerans | 0.673** | GABA | 0.417** | Bacteroides_thetaiotaomicron | 0.583** |
| Veillonellaceae_bacterium_canine_oral_taxon_211 | 0.485** | Ruminococcaceae_bacterium_GD6 | 0.602** | Megasphaera_elsdenii | 0.405** | Dialister_pneumosintes | 0.507** |
| Kallipyga_sp._GM4 | 0.464** | Dorea_longicatena | 0.575** | Parabacteroides_distasonis | 0.394** | Ruminococcaceae_bacterium_GD6 | 0.482** |
| bacterium_OL_1 | 0.450** | Clostridium_scindens | 0.538** | Clostridium_lavalense | 0.391** | GABA | 0.436** |
| Propionate | 0.436** | Escherichia_coli | 0.514** | bacterium_OL_1 | 0.370** | Dialister_micraerophilus | 0.410** |
| Pyramidobacter_piscolens | 0.431** | Erysipelatoclostridium_ramosum | 0.502** | Enterococcus_durans | 0.370** | Dorea_longicatena | 0.398** |
| Lactobacillus_reuteri | 0.419** | Clostridiales_bacterium_10_3b | 0.471** | Bacteroides_thetaiotaomicron | 0.367** | Megasphaera_micronuciformis | 0.396** |
| Butyrate | 0.417** | Clostridiales_bacterium_60_7e | 0.464** | Pyramidobacter_piscolens | 0.347* | Anaerostipes_hadrus | 0.383** |
| Eubacterium_ramulus | 0.408** | Bacteroides_thetaiotaomicron | 0.460** | Clostridiales_bacterium_S5_A14a | 0.344* | Blautia_producta | 0.364** |
| Dialister_succinatiphilus | 0.383** | Intestinimonas_butyriciproducens | 0.416** | Peptoniphilus_sp._BV3AC2 | 0.342* | Intestinimonas_butyriciproducens | 0.325* |
| Campylobacter_hominis | 0.377** | Clostridium_spiroforme | 0.413** | Collinsella_aerofaciens | 0.338* | Parabacteroides_distasonis | 0.323* |
| Enterococcus_durans | 0.368** | Gordonibacter_urolithinfaciens | 0.404** | Kallipyga_sp._GM4 | 0.326* | Clostridiales_bacterium_10_3b | 0.315* |
| Prevotella_denticola | 0.346* | Ruminococcus_sp._5_1_39BFAA | 0.383** | Eubacterium_ramulus | 0.319* | Bacteroides_ovatus | 0.315* |
| Clostridiales_bacterium_S5_A14a | 0.346* | Parabacteroides_distasonis | 0.377** | Dorea_longicatena | 0.319* | Gordonibacter_urolithinfaciens | 0.309* |
| Megasphaera_micronuciformis | 0.322* | Clostridium_lavalense | 0.370** | Clostridium_scindens | 0.294* | Escherichia_coli | 0.284* |
| Prevotella_timonensis | 0.320* | butyrate_producing_bacterium_L2_10 | 0.337* | Anaerococcus_vaginalis | 0.293* | Bacteroides_cellulosilyticus | -0.293* |
| intestinal_bacterium_CG19_1 | 0.316* | Bacteroides_ovatus | 0.332* | Dielma_fastidiosa | 0.285* | Bacteroides_stercoris | -0.308* |
| Porphyromonas_somerae | 0.305* | Clostridium_sp._GD3 | 0.301* | Campylobacter_hominis | 0.281* | Alistipes_sp._N15.MGS_157 | -0.310* |
| Acinetobacter_lwoffii | 0.304* | Clostridium_symbiosum | 0.297* | Veillonellaceae_bacterium_canine_oral_taxon_211 | 0.279* | Anaerotruncus_sp._MT15 | -0.314* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Bacteroides_fragilis | 0.299* | Bacteroides_clarus | 0.293* | Alistipes_indistinctus | -0.283* | Bacteroides_coprophilus | -0.327* |
| Clostridiales_bacterium_60_7e | -0.289* | Anaerostipes_hadrus | 0.290* | Clostridium_sp._K4410.MGS_306 | -0.306* | Haemophilus_parainfluenzae | -0.329* |
| Sutterella_wadsworthensis | -0.290* | Dielma_fastidiosa | 0.289* | bacterium_YE57 | -0.308* | Bacteroides_coprocola | -0.352* |
| Ruminococcus_bicirculans | -0.293* | Bifidobacterium_adolescentis | 0.281* | Prevotella_corporis | -0.316* | bacterium_YE57 | -0.353* |
| Sanguibacteroides_justesenii | -0.306* | Prevotella_corporis | -0.294* | Bacteroides_stercoris | -0.319* | Alistipes_indistinctus | -0.356* |
| Erysipelatoclostridium_ramosum | -0.321* | bacterium_YE57 | -0.295* | Haemophilus_parainfluenzae | -0.335* | Clostridium_sp._K4410.MGS_306 | -0.358* |
| Bacteroides_clarus | -0.336* | Bacteroides_plebeius | -0.309* | Bacteroides_coprocola | -0.364** | Ruminococcus_bicirculans | -0.371** |
| Clostridium_aldenense | -0.392** | Haemophilus_parainfluenzae | -0.315* | Roseburia_inulinivorans | -0.496** | Others | -0.374** |
| Ruminococcus_sp._5_1_39BFAA | -0.394** | Bacteroides_coprocola | -0.319* | Ruminococcus_bicirculans | -0.538** | Ruminococcus_sp._UNK.MGS_30 | -0.427** |
| | | Clostridium_sp._K4410.MGS_306 | -0.320* | Roseburia_intestinalis | -0.541** | Roseburia_inulinivorans | -0.499** |
| | | Others | -0.426** | Ruminococcus_sp._UNK.MGS_30 | -0.544** | Roseburia_intestinalis | -0.560** |
| | | Ruminococcus_sp._UNK.MGS_30 | -0.464** | | | | |
| | | Alistipes_indistinctus | -0.494** | | | | |
| | | Roseburia_intestinalis | -0.499** | | | | |
| | | Roseburia_inulinivorans | -0.500** | | | | |

## 5.1 SPSS (Pearson correlation) analysis correlating bacterial profiling at specie level with GABA, Acetate, butyrate and propionate production for gut model.

| Pearsons Correlations (N=50) | GABA | Pearsons Correlations (N=50) | Acetate | Pearsons Correlations (N=50) | Butyrate | Pearsons Correlations (N=50) | Propionate |
|---|---|---|---|---|---|---|---|
| *Bacteroides_thetaiotaomicron* | 0.679** | *Propionate* | 0.852** | *Propionate* | 0.836** | *Acetate* | 0.852** |
| *Dialister_pneumosintes* | 0.669** | *Butyrate* | 0.762** | *Acetate* | 0.762** | *Butyrate* | 0.836** |
| *Peptoniphilus_sp._BV3AC2* | 0.531** | *Blautia_producta* | 0.677** | *Dorea_formicigenerans* | 0.546** | *Dorea_formicigenerans* | 0.642** |
| *Megasphaera_elsdenii* | 0.519** | *Dorea_formicigenerans* | 0.673** | *GABA* | 0.417** | *Bacteroides_thetaiotaomicron* | 0.583** |
| *Veillonellaceae_bacterium_canine_oral_taxon_211* | 0.485** | *Ruminococcaceae_bacterium_GD6* | 0.602** | *Megasphaera_elsdenii* | 0.405** | *Dialister_pneumosintes* | 0.507** |
| *Kallipyga_sp._GM4* | 0.464** | *Dorea_longicatena* | 0.575** | *Parabacteroides_distasonis* | 0.394** | *Ruminococcaceae_bacterium_GD6* | 0.482** |
| *bacterium_OL_1* | 0.450** | *Clostridium_scindens* | 0.538** | *Clostridium_lavalense* | 0.391** | *GABA* | 0.436** |
| *Propionate* | 0.436** | *Escherichia_coli* | 0.514** | *Enterococcus_durans* | 0.370** | *Dialister_micraerophilus* | 0.410** |
| *Pyramidobacter_piscolens* | 0.431** | *Erysipelatoclostridium_ramosum* | 0.502** | *bacterium_OL_1* | 0.370** | *Dorea_longicatena* | 0.398** |
| *Lactobacillus_reuteri* | 0.419** | *Clostridiales_bacterium_10_3b* | 0.471** | *Bacteroides_thetaiotaomicron* | 0.367** | *Megasphaera_micronuciformis* | 0.396** |
| *Butyrate* | 0.417** | *Clostridiales_bacterium_60_7e* | 0.464** | *Pyramidobacter_piscolens* | 0.347* | *Anaerostipes_hadrus* | 0.383** |
| *Eubacterium_ramulus* | 0.408** | *Bacteroides_thetaiotaomicron* | 0.460** | *Peptoniphilus_sp._BV3AC2* | 0.342* | *Blautia_producta* | 0.364** |
| *Dialister_succinatiphilus* | 0.383** | *Intestinimonas_butyriciproducens* | 0.416** | *Collinsella_aerofaciens* | 0.338* | *Intestinimonas_butyriciproducens* | 0.325* |
| *Campylobacter_hominis* | 0.377** | *Clostridium_spiroforme* | 0.413** | *Kallipyga_sp._GM4* | 0.326* | *Parabacteroides_distasonis* | 0.323* |
| *Enterococcus_durans* | 0.368** | *Gordonibacter_urolithinfaciens* | 0.404** | *Bacteroides_stercoris* | 0.319* | *Bacteroides_ovatus* | 0.315* |
| *Prevotella_denticola* | 0.346* | *Ruminococcus_sp._5_1_39BFAA* | 0.383** | *Eubacterium_ramulus* | 0.319* | *Clostridiales_bacterium_10_3b* | 0.315* |
| *Clostridiales_bacterium_S5_A14a* | 0.346* | *Parabacteroides_distasonis* | 0.377** | *Clostridium_scindens* | 0.294* | *Gordonibacter_urolithinfaciens* | 0.309* |
| *Megasphaera_micronuciformis* | 0.322* | *Clostridium_lavalense* | 0.370** | *Anaerococcus_vaginalis* | 0.293* | *Escherichia_coli* | 0.284* |
| *Prevotella_timonensis* | 0.320* | *butyrate_producing_bacterium_L2_10* | 0.337* | *Dielma_fastidiosa* | 0.285* | *Bacteroides_cellulosilyticus* | -0.293* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *intestinal_bacterium_CG19_1* | 0.316* | *Bacteroides_ovatus* | 0.332* | *Campylobacter_hominis* | 0.281* | *Bacteroides_stercoris* | -0.308* |
| *Porphyromonas_somerae* | 0.305* | *Clostridium_sp._GD3* | 0.301* | *Veillonellaceae_bacterium_canine_oral_taxon_211* | 0.279* | *Alistipes_sp._N15.MGS_157* | -0.310* |
| *Acinetobacter_lwoffii* | 0.304* | *Clostridium_symbiosum* | 0.297* | *Alistipes_indistinctus* | -0.283* | *Anaerotruncus_sp._MT15* | -0.314* |
| *Bacteroides_fragilis* | 0.299* | *Bacteroides_clarus* | 0.293* | *Clostridium_sp._K4410.MGS_306* | -0.306* | *Bacteroides_coprophilus* | -0.327* |
| *Clostridiales_bacterium_60_7e* | -0.289* | *Anaerostipes_hadrus* | 0.290* | *bacterium_YE57* | -0.308* | *Haemophilus_parainfluenzae* | -0.329* |
| *Sutterella_wadsworthensis* | -0.290* | *Dielma_fastidiosa* | 0.289* | *Prevotella_corporis* | -0.316* | *Bacteroides_coprocola* | -0.352* |
| *Ruminococcus_bicirculans* | -0.293* | *Bifidobacterium_adolescentis* | 0.281* | *Dorea_longicatena* | -0.319* | *bacterium_YE57* | -0.353* |
| *Sanguibacteroides_justesenii* | -0.306* | *Prevotella_corporis* | -0.294* | *Haemophilus_parainfluenzae* | -0.335* | *Alistipes_indistinctus* | -0.356* |
| *Erysipelatoclostridium_ramosum* | -0.321* | *bacterium_YE57* | -0.295* | *Bacteroides_coprocola* | -0.364** | *Clostridium_sp._K4410.MGS_306* | -0.358* |
| *Bacteroides_clarus* | -0.336* | *Bacteroides_plebeius* | -0.309* | *Roseburia_inulinivorans* | -0.496** | *Ruminococcus_bicirculans* | -0.371** |
| *Clostridium_aldenense* | -0.392** | *Haemophilus_parainfluenzae* | -0.315* | *Ruminococcus_bicirculans* | -0.538** | *Others* | -0.374** |
| *Ruminococcus_sp._5_1_39BFAA* | -0.394** | *Bacteroides_coprocola* | -0.319* | *Roseburia_intestinalis* | -0.541** | *Ruminococcus_sp._UNK.MGS_30* | -0.427** |
| | | *Clostridium_sp._K4410.MGS_306* | -0.320* | *Ruminococcus_sp._UNK.MGS_30* | -0.544** | *Roseburia_inulinivorans* | -0.499** |
| | | *Others* | -0.426** | | | *Roseburia_intestinalis* | -0.560** |
| | | *Ruminococcus_sp._UNK.MGS_30* | -0.464** | | | | |
| | | *Alistipes_indistinctus* | -0.494** | | | | |
| | | *Roseburia_intestinalis* | -0.499** | | | | |
| | | Roseburia_inulinivorans | -0.500** | | | | |

## 2.1 Isolated LAB strains from fermented olives

| | **Strains** | **code** |
|---|---|---|
| 1A | *L. paracasei* | E94 |
| 2A | *L. pentosus* | E104 |
| 3A | *L. paracasei* | E93 |
| 4A | *L. plantarum* | E10 |
| 5A | *L. pentosus* | 108 |
| 6A | *L. pentosus* | E97 |
| 7A | *L. pentosus* | B281 |
| 8A | *L. plantarum* | E69 |
| 9A | *L. rhamnosus* | GG ATCC S 3103 |
| 10A | *L. plantarum* | B282 |
| 11A | *L. casei shirota* | |