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# Determination of the Effects of Different Maillard Reaction Products on the Taxonomic Composition of the Gut Microbiota

Nesreen Hamdan ALJahdali
*University of Arkansas, Fayetteville*

Determination of the Effects of Different Maillard Reaction Products on the Taxonomic Composition of the Gut Microbiota

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by

Nesreen ALJahdali
College of Education and Science
Bachelor of Science in Biology, 2002
King Abdul Aziz University
Master of Science in Biology-Animal Ecology, 2007

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University of Arkansas

This dissertation is approved for recommendation to the Graduate Council.

_____
Dr. Franck Carbonero
Dissertation Director

_____
Dr. Steve Ricke
Committee Member

_____
Dr. Sami Dridi
Committee Member

_____
Dr. Charles Rosenkrans
Committee Member

ABSTRACT:

The Maillard Reaction (MR) is a non-enzymatic chemical reaction which results in linkage between the amino group of amino acids and the carbonyl group of reduced sugars. This reaction generates Maillard reaction products (MRPs) which are not present naturally in foods, and are responsible for a range of colors, odors, flavors, and other sensory properties. Conflicting reports of MRPs impacts on human health are probably due to the fact that bioconversion of these digestible molecules by the gut microbiota has been marginally taken into account. This study aimed to determine the effects of different MRPs on rodent's gut microbiota through16S rRNA amplicon sequencing over three different studies. Study 1 focused on the impact of $N^{\varepsilon}$Carboxymethyllysine (CML) on the composition of mice gut microbiota and potential association with severity of experimental colitis. Study 2 focused on the impact of bread melanoidins on the composition of healthy and experimental colitis mice gut microbiota. Study 3 focused on the impact of consumption of increasing amounts of malt melanoidins on mice gut microbiota. It was found that CML induced limited changes in gut microbiota profiles of healthy mice, but was found to significantly relieve the bacterial dysbiosis imparted by one (but not the other) inflammation-inducing chemical, especially the Proteobacteria bloom. Bread crust model (high in melanoidins) showed significant decreases of *Bacteroides* spp. and Enterobacteriaceae, while it increased *Faecalibacterium* spp. Also, bread crust model limited to increase Enterobacteriaceae in colitis model. High amounts of malts rich melanoidins rapidly and persistently led to significantly different gut microbiota profiles. There was a trend for decrease of *Lactobacillus* and *Ruminococcus* and increase of *Akkermansia* and *Bifidobacterium* with higher amounts of dietary melanoidins. We concluded that CML and melanoidins are not detrimental in terms of their impact on the gut microbiota, and that they may even have prebiotic properties.

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GENERAL INTRODUCTION:

A clarification in terms of microbes used in the field of science and medicine is necessary before delving into the literature. Microbiome, the most commonly used term, refers to the whole genomic content of microorganisms in a given environment. Microbiota should be used when only the taxonomy of the microorganisms is surveyed. Microflora has been and is still used, most often by clinicians, but it is an incorrect term as microorganisms cannot be considered as plants. Metabolome is used to refer to the total composition of metabolites present in organs and fluids, typically measure in blood or urine (less often in fecal samples). Evidence is rapidly emerging that the gut microbiota has a strong association with health. The gut microbiota has been demonstrated to play an important role in the gut maturation, development of innate immunity, production of vitamins, and dietary energy harvest. The human body hosts up to 100 trillion ($10^{14}$) microbes, with the majority residing in the gastrointestinal tract (GIT) of humans and animals. This complex community consists of taxa from across the tree of life, bacteria, Archaea, eukaryotes (fungi and protozoa), and viruses, greatly impacting human physiology (Walter & Ley, 2011).

The majority of microbiota in the GIT are bacteria, especially anaerobic bacteria. The bacterial phyla that are consistently identified in human stool or intestinal bioptic samples are Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia, and Actinobacteria. There is a strong difference in microbial load between upper GI with amount $10^{2}$-$10^{4}$ cells/ ml, and the lower GI with increased number in the small intestine ($10^{4}$-$10^{5}$ cells/ml) but especially in the colon (large intestine, $10^{11}$ cells/ml (Walter & Ley, 2011). The generally symbiotic nature between the host and microbiota are described in terms of nutrient exchange. The function of gut microbiota is involved in energy harvest and storage, as well as in a variety of metabolites (Gill et al., 2006). Gut

microbiota plays an essential role in degrading undigested dietary elements and producing a vast array of metabolites, which can influence the benefit of the epithelium tissue in the GIT as well as the immune system (Martin, Miquel, Ulmer, Langella, & Bermudez-Humaran, 2014; Nicholson et al., 2012). It has been shown that the composition of the microbiota is relatively stable within healthy adult individuals through time (Caporaso et al., 2011). However, among the environmental and genetic factors, dietary habits play an important role in alteration of the gut microbiota composition so that the colonic microbiota are linked in the context of health and disease of human and animal.

The variation in the gut microbiota has been associated with long-term or short-term dietary habits. To confirm this hypothesis, the prevalence of Bacteroidetes is associated with animal-based diet. In contrast, the dominance of *Prevotella* is associated with carbohydrates-based diet (Wu et al., 2011). Additionally, in an animal-based diet, the prevalence of bile-tolerant bacteria, such as *Alistipes*, *Bilophila,* and *Bacteroises* increased whereas the dominance of metabolizing plant polysaccharide microorganism decreased (David et al., 2014). The Western diet- typically is described by higher consumption of red meat, animal fats, low fiber, and high sugar-has become an increasingly popular diet choice in the last decade. Different food preparation methods, such as roasting, frying, and toasting, generate Maillard reaction products (MRPs). Maillard reaction (MR) is non-enzymatic modification occurring between the carbonyl group of reducing sugar molecules with the amino group of amino acids, which produce low-weight and high-weight molecules that are not naturally present in foods. These molecules have been found in more than 200 food items within the Western diet and are responsible for the aromas, colors, and tastes of foods (Goldberg et al., 2004; Hull, Woodside, Ames, & Cuskelly, 2012). For example, coffee and bread are the major source of melanoidins, which generate in the last stage of the MR (Fogliano & Morales,

2011). Fried and broiled meat are rich in advanced glycation end products (AGEs), which generate in the intermediate stage of the MR (Van Nguyen, 2006). Data from metabolic transit found that the dietary of $N^{\varepsilon}$Carboxymethyllysine (CML) and melanoidins recovered in urine and feces, but the majority of them are not yet accounted for (Faist & Erbersdobler, 2001).

Because MRPs are largely present in the Western diet, they may be considered as one of the dietary elements that have both a beneficial and detrimental impact on human health, specifically through the gut microbiota. CML was found degraded by gut microbiota through *in vitro* studies (Hellwig et al., 2015; Seiquer, Rubio, Jesus Peinado, Delgado-Andrade, & Pilar Navarro, 2014). In addition to CML, melanoidins have been used as a carbon source and increased growth rates of some bacteria, such as *Bifidobacteria*, *Bacteroides* and *prevotella* (Borrelli & Fogliano, 2005; Reichardt, Gniechwitz, Steinhart, Bunzel, & Blaut, 2009). Because the impact of MRPs on gut microbiota have been studied most commonly *in vitro* models, there still remain gaps of knowledge about the effect of MRPs on gut microbiota in an *in vivo* study.

Objective:

The main objectives of this study were to determine the effect of different Maillard reaction products on the composition of mice gut microbiota by sequencing 16S rRNA gene. The objective was aimed at testing three different studies:

1- To determine the effect of NƐCarboxymethyllysine (CML) on the composition of mice gut microbiota and potential association with severity of experimental colitis.
2- To determine the effect of bread melanoidins on the composition of mice gut microbiota and potential positive modulation of the microbiota profile associated with experimental colitis.

3- To determine the effect of increased concentration of melanoidin-rich malts on the composition of mice gut microbiota and potential prebiotic effects.

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CHAPTER ONE

REVIEW OF THE LITERATURE

# Impact of Maillard Reaction products on Nutrition and Health: Current knowledge and need to understand their fate in the human digestive system

ALJahdali Nesreen[1] and Carbonero Franck[1,2*]

[1]Cell and Molecular Biology Program; University of Arkansas, AR, USA

[2]Department of Food Science; University of Arkansas, AR, USA

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## 1.1 Abstract:

The Maillard Reaction (MR) is a non-enzymatic chemical reaction which results in the linkage between the amino group of amino acids and the carbonyl group of reduced sugars. MR products (MRPs) are common components of processed foods, mainly as a result of heating, especially in the Western diet. MRPs are classified as into three stages: the initial, intermediate, and final stage, indicative of increased complexity and size, incurring different flavor, aroma, and texture. MRPs presence are known to reduce the nutritional quality of foods, particularly by reducing protein digestibility. Early reports have linked MRPs, especially advanced glycation end-products (AGEs) present in high concentration in the typical Western diet, to health conditions and diseases. However, conflicting data have since been reported, and only a few (acrylamide, heterocyclic amines and 5-Hydroxymethylfurfural) MRPs have documented potential toxic or carcinogenic effects. High molecular weight MRPs are not available for direct absorption in the higher gastrointestinal tract, and are thus mostly metabolized by resident colonic microbes. MRPs have been the subject of sparse research interest in comparison with other non-digestible dietary elements. In this review, we outline the state of knowledge on MRPs in nutrition and health, and highlight the need to develop the limited knowledge on their impact on the gut microbiota and which metabolites derive from MRPs fermentation.

**Keywords**: Maillard reaction products, Gut microbiota, Metabolomics, Advanced glycation end-products

## 1.2 Introduction:

Western diet is becoming the dominant diet worldwide, and this trend is suspected to play an important role in the rise of western diseases. Western diet is characterized by higher intakes of red meat, fast foods, high-fat dairy products, fried and baked foods, high-sugar drinks, and a reduced intake of fibers and whole grains. While higher intake of simple sugars and fat are well known to increase disease and health condition risks, there are also specific dietary elements that have been reported as detrimental. In this review, we will focus on Maillard Reaction Products (MRP), a relatively large class of molecules formed by linkage between carbohydrates and proteins/peptides. MRPs are known to occur in high levels in typical Western diet foodstuffs resulting from different food preparation methods, such as roasting, frying, and toasting. While early studies on MRPs have pointed to their role as biomarkers of Western diet consumption and potential correlation with disease risk; there is currently no consensus on the role of MRPs in human health.

Although it has been known for decades that a symbiotic relationship exists between the host and microbiota, it is only recently that analytic tools have allowed for precise characterization of both microbiota members and their metabolites. It is now well established that colonic microbes play an essential role in degrading undigested dietary elements and produce a vast array of metabolites. Diet-microbiota interactions are increasingly investigated in the context of health and disease (human and animal), with a focus on cancer, inflammatory and metabolic diseases, obesity and more recently cognition and neurology. Surprisingly, MRPs and MRPs-rich food interaction with the gut microbiota have received little attention from researchers in comparison with other dietary elements.

The purpose of this review is to outline the current knowledge on MRPs in the context of nutrition and health, and provide an overview of the scarce knowledge on metabolic impacts, microbiota interaction and metabolomics. We will conclude by summarizing the aspects for which extensive knowledge is available, and state the research directions that need to be undertaken to complete our knowledge of MRPs metabolic impacts.

## 1.3 The Chemistry of Maillard Reaction Products:

### 1.3.1 Maillard reaction in food

The Maillard reaction (MR) was first described by Louis Camille Maillard in 1912, as the non-enzymatic chemical reaction between the carbonyl group of reducing sugar molecules with the amino group of amino acids occurring during processing and storage of foods. This reaction depends on physical parameters, such as heating, hydration, pH, and $NH_2$ requirements in order to form complex compounds that are not naturally in foods and are responsible for a range of colors, odors, flavors, and palatability. Thus, these molecules have positive or negative biological actions.

MR is divided into three stages: initial, intermediate, and final stage (Hodge. 1953) as described in Figure1.1. In the initial stage, colorless products such as sugar-amine condensation and Amadori rearrangement products are produced. In the intermediate stage, yellow or colorless (with strong UV absorption) compounds are produced, including 5-Hydroxymethylfurfural, reductone, and dicarbonyl compounds. In the final stage, brown color compounds are produced, such as melanoidins. The coloration occurs during heat pyrolysis of sugar, due to a pH reaction on the carbonyl group of sugar, while amino acids are not directly responsible for coloration (Adrian. 1974). The characteristic color in foodstuff, such as coffee, malt, bread, cocoa, and other roasted foods is the result of melanoidins, which are brown nitrogen-containing high molecular weight

pigments (Bastos et al. 2012). In addition to desirable color, the intermediate and final stages are the most important for developing flavor and aroma, through Strecker degradation (Somoza. 2007, Ames. 1990). MR can also affect the texture of food through protein cross-linking (Gerrard. 2002).

### 1.3.2 Generation of Maillard Reaction Products in vivo

In this review, we will focus on dietary MRPs. However, it is worth noting that MRPs have also been shown to be produced endogenously in humans. The knowledge on endogenous MR is reviewed extensively in Tessier (2010). The first report of MR *in vivo* was the glycation of aging proteins (Monnier and Cerami. 1981). In biological systems, this reaction is mainly implicated in protein modification, and divided into early and advance reaction stages. In the early stage, the formation of the Schiff base occurs, which is the interaction between the amine group of proteins with the reducing sugar, which generates α-dicarbonely compounds, or rearranges into the Amadori product. In the advance stage, the Amadori product undergoes rearrangements, which forms advanced glycation end products (AGEs) (Brownlee et al. 1984). The AGEs that have been detected in tissue protein are $N^{\varepsilon}$Carboxymethyllysine (CML), Pentosidins, and Glucosepane, and CML was the first AGEs isolated and characterized *in vivo* (AHMED et al. 1986). The receptor of AGEs (RAGE) is a multi-ligand member of a cell (Schmidt et al. 2000). Previous studies demonstrated that CML/RAGE plays an important role in the induction of a calcification cascade in diabetes (Wang et al. 2016). Thus, AGEs are known as metabolic products of glucose toxicity and play a significant role in the development of metabolic diseases (Wang et al. 2012).

### 1.3.3 Important Maillard Reaction Product Molecules

Evidence indicates that the most important Maillard reaction products in common diets are $N^{\varepsilon}$Fructoselysine (furosine), 5-Hydroxymethylfurfural (HMF), acrylamide, heterocyclic amines, advanced glycation end products (AGEs), and melanoidins. They all impact the nutritional quality of foodstuffs and biological systems either positively or negatively, as reviewed by Tuohy et al. (2006). Table (1.1) summarizes the example of MRPs content of commonly consumed foods.

#### 1.3.3.1 *$N^{\varepsilon}$Fructoselysine (Furosine) (FL)*:

The α-amino and Ɛ-amino group of lysine interact with reducing sugar, such as glucose, fructose, and maltose to form glycosylamine that undergo Amadori rearrangement products (ARP) in the early stage of the Maillard reaction (Hodge. 1953). Amadori products are measured as $N^{\varepsilon}$fructoselysine because it was the first MRPs identified in foods, and is used as an indicator of the nutritional quality of foods. Moreover, Furosine (FL) amount is used to estimate protein damage caused by heating in the initial stage of MR in cereal products, such as pasta and bread (Erbersdobler and Somoza. 2007, Delgado-Andrade et al. 2005, Resmini et al. 1991). For example, low FL values may indicate a decrease in pasta quality due to exposure to low temperatures (Garcia-Banos et al. 2004). Temperature and time play an important role in the rise or decline of FL content in foods. For example, FL levels of soybean was high in extrusion treatments (66.55 µg/g) at 140 °C for 20-30s, followed by infrared heating (63.93 µg/g) at 110 °C for 50s, and microwave heating (56.07 µg/g) at 115C°for 5min (Zilic et al. 2014). Heating foods for a long time decreases the level of FL which gives rise to other products in the intermediate stage (Erbersdobler and Faist. 2001).

1.3.3.2 *5-Hydroxymethylfurfural (HMF)*:

5-Hydroxymethylfurfural (HMF) is produced in the intermediate stage of the Maillard reaction, and it forms in carbohydrate-rich food during acid-catalyzed dehydration of the Schiff base of furfural (Hodge. 1953) (Figure 1.1). HMF is a widely used marker of the nutritional quality of foods, such as baked diets and coffee, and it is not present in raw and fresh foods (Erbersdobler and Somoza. 2007). The concentration of HMF increases as thermal treatments or storage time of foodstuffs increase. Specifically, a positive correlation has been found between HMF content and the development of browning color so that reducing the heating period might be possible to reduce the concentration of HMF (Capuano et al. 2008). In addition to temperature, increasing pH plays an important role in decreasing the quantity of HMF in bakery products (Gokmen et al. 2007). Moreover, the type of sugar results in various quantities of HMF molecules. For example, hexose produces 4 to 5 times more HMF than pentose in baked foods. In addition to the type of sugars, the presence of certain amino acids, such as leucine, valine, and methionine can be linked to the concentration of HMF molecules in food products (Adrian. 1974). HMF is also formed through the caramelization of sugars (Capuano and Fogliano. 2011). HMF has been found in different quantities in various foods. The concentration of HMF in dried fruits and caramel are high, but bakery foods and coffee are the major sources of HMF intake (Capuano and Fogliano. 2011, Murkovic and Pichler. 2006). It has been reported that coffee is the main source of HMF; the concentration of HMF in natural, blend, roasted and soluble coffee were 110, 625, 1734, 2480 mg HMF/kg, respectively (Arribas-Lorenzo and Morales. 2010).

1.3.3.3 *Acrylamide*:

Acrylamide, which is generated during the intermediate stage of the Maillard reaction, results from the interaction between asparagine and reducing sugars, such as fructose and glucose, in heat treated bakery products and starchy foods (Hodge. 1953) (Figure 1.1). A diversity of chemical pathways lead to the formation of acrylamide in carbohydrate-rich foods (Granvogl and Schieberle. 2006, Granvogl and Schieberle. 2007). However, the major pathways are through Amadori products that degrade to form dicarbonyl compounds, which react with asparagine via Strecker degradation; or by the interaction of reducing sugar and asparagine to form the Schiff base without Amadori product (Granvogl and Schieberle. 2007, Granvogl and Schieberle. 2006). Like HMF, the formation of acrylamide is dependent on the type and concentrations of sugars, amino acids, temperature, and time. A positive correlation has been found between acrylamide levels and heating-time during baking of biscuits at 200 degrees °C and in potato chips that were fried at more than 248 °F (Nguyen et al. 2016, Tareke et al. 2002). Moreover, the interaction between glucose and asparagine generated the highest concentration of acrylamide, compared to fructose and asparagine (Capuano and Fogliano. 2011). Indeed, adding asparginase might control acrylamide content in potato products (Zyzak et al. 2003). Unlike microwaved and boiled foods, the highest acrylamide concentration is formed through roasting, frying, and baking methods. The highest level of acrylamide was found in fried potato products. For instance, the average level of acrylamide found in potato crisps was 628 µg/kg, compared to biscuits, bread, and coffee, which were 317 µg/kg, 136 µg/kg, and 253 µg/kg, respectively (Capuano and Fogliano. 2011).

1.3.3.4 *Heterocyclic amines (HCAs)*:

Heterocyclic amines (HCAs), produced in the intermediate stage of the Maillard reaction, result from the reaction between reducing sugar, amino acids, and their precursor creatine (a nitrogenous organic acid found naturally in muscles). To illustrate, the fragmentation of Amadori products can form various dicarbonyl compounds that can act with amino acids and creatine to form HCAs (JAGERSTAD et al. 1991, Tuohy et al. 2006) (Figure 1.1). Increasing temperature and time play an important role in generating HCAs, which are mainly found in muscle foods, such as beef, pork, chicken, and fish. The most common of HCAs found and studied in foods are 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP), 2-amino-3-methyl-imidazo [4,5-f]quinoline (IQ), 2-amino-3-methylimidazo [4,5-f]quinoxaline (IQx), 2-amino-3,4-dimethylimidazo [4,5-f]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo [4,5-f]quinoxaline (MeIQx) and 2-amino-3,4,8-trimethyl-imidazo [4,5-f]quinoxaline (DiMeIQx) (Knize et al. 1994,Puangsombat et al. 2012).

The levels of HCAs in cooked meat depends on the type of meat and meat preparation methods. It has been reported that well done cooked beef had a higher concentration of HCAs, compared to medium cooked beef. Moreover, the highest level of total content of HCAs was quantified in fried bacon (17.59 ng/g), compared to fried pork (13.91 ng/g), fried beef (8.92 ng/g), or fried chicken (7.06 ng/g) (Puangsombat et al. 2012). In addition to total content of HCAs, high concentrations of PhIP were found in fried Tilapia (10.89 ng/g), followed by MeIQx (4.00 ng/g) and DiMeIQx (3.57 ng/g) in fried bacon, but IQ was not identified (Puangsombat et al. 2012). However, another study found that the high levels of IQ was10.5 ng/g in well-done fried bacon, which had high content of fat (Johansson and Jägerstad. 1994). It has also been reported that fried

meat produced the highest concentration of HCAs, compared to baked meat (Puangsombat et al. 2012).

1.3.3.5 *Advanced Glycation End products (AGEs)*:

The interaction between glucose and protein or glucose and lipid generate advanced glycation end products (AGEs) that are also known as advanced Maillard reaction products (Obrien and Morrissey. 1989). AGEs are generated in the intermediate stage of the Maillard reaction. The degradation of Amadori products generate reactive dicarbonyl compounds that react further with amino acids to form irreversible and highly stable advance glycation end products (AGEs) (Tuohy et al. 2006,Cho et al. 2007) (Figure 1.1). AGEs are also produced endogenously through glycation metabolic pathways (Monnier and Cerami. 1981). It has been found that Western diet is rich in AGEs, so this review concentrates on food-derived AGEs that have been detected and measured in more than 200 food items (Goldberg et al. 2004). The highest content of AGEs was found in fat food items, such as butter with a mean of 100± 19 kilounits (kU)/g, compared to carbohydrate diet that contained the lowest levels of AGEs with a mean of 3.4±1.8 k U/g (Goldberg et al. 2004). Moreover, the heating period and preparation methods appear to be more critical to form AGE. For example, the highest content of AGE was found in grilled foods at temperatures of 230 °C for short time, compared to boiled foods at 100°C for long periods (Goldberg et al. 2004). There are many types of AGEs, and the most commonly studied are $N^{\varepsilon}$Carboxymethyllsine (CML) (non-cross-linking), pyrraline and pentosidine (cross-linking), which are most widely used as indicators of the nutritional quality of foodstuffs (Erbersdobler and Somoza. 2007).

$N^{\varepsilon}$Carboxymethyllsine (CML) is the most important bioactive marker of MRPs and a commonly measured AGE not only in food items (Goldberg et al. 2004) but also in biospecimens

(Uribarri et al. 2003, Hofmann et al. 2002, Tessier et al. 2016). CML can be produced by the reaction of the carbonyl group of glyoxal from dicarbonyl componds with an epsilon-amino group of the lysine or by Amadori rearrangement products that act as a precursor of CML (HODGE. 1953, Tuohy et al. 2006). Besides furosine, CML was found to be a useful indicator of protein damage during the late stage of the Maillard reaction (Van Nguyen. 2006). Hull *et al* (2012) determined the CML content in 257 foods that are typically consumed in Western style diets.

1.3.3.6 *Melanoidins*:

Melanoidins, which are the final products of MR, are heterogeneous, insoluble, nitrogen-containing high molecular weight molecules that are generated in the advanced stage of MR. Melanoidins are formed by dehydration, rearrangements, isomerization, and condensation of low molecules of MRPs formed in the intermediate stage (Hodge. 1953). To illustrate, during the intermediate stage, dicarbonyl compounds, aldehydes, and furfural are generated, which react with each other to form aldol condensation products that react with amino acid to give rise to low molecular weights of MRPs, leading to the high molecular weights of melanoidins (HODGE. 1953) (Figure 1.1). Temperature and time appear to be significant factors affecting molecular weight, while pH plays an essential role in the chemical structure of melanoidins (Wang et al. 2011). The color of melanoidins that are found in coffee, malt, bread crust, cocoa, and honey, derive from the polymerization of MRPs (Hofmann. 1998, Hofmann. 1999). The highest amount of melanoidins was found in sourdough loaves (30 g per 100 g of crust), compared to soluble coffee (22.8 g per 100 g of coffee), but the quantity of melanoidins depends on the type of bread and the degree of roasting in coffee (Fogliano and Morales. 2011).

## 1.4 Maillard Reaction Products Impact on Nutrition and Health

### 1.4.1 Consequences of the Maillard Reaction in Nutrition:

Western diet, also known as the American standard diet, is characterized by higher intakes of red meat, high-fat dairy products, fried and baked foods, high-sugar drinks, and a reduced intake of fibers and whole grains. MRPs, which are not naturally present in foods, are common in the Western diet (Hull et al. 2012). More than 200 staple food items of the typical Western diet contain measurable MRPs. These MRPs are the result of different food preparation methods, such as roasting, frying, and toasting, which are responsible for the aromas, colors, and tastes of foods (Goldberg et al. 2004, Hull et al. 2012,Zilic et al. 2014). For example, coffee and bread are the major source of Melanoidins (Fogliano and Morales. 2011), fried chicken and broiled beef are rich in AGEs (Van Nguyen. 2006), and HCAs are found in high concentration in cooked meat (Tamanna and Mahmood. 2015).

The typical exposure to several dietary MRPs has been reported by different survey-based studies. The estimation of dietary exposure to HMF from coffee was 5.26 mg HMF/day (Arribas-Lorenzo and Morales. 2010). The mean daily HCAs intake from meat products in a typical western diet was estimated at 450 ng per day$^{-1}$, including mainly PhIP, MeIQx and DiMeIQx (Keating et al. 1999). The average daily intake level of HCAs in the Malaysia population was 553.7 ng per capita day, and the highest level was PhIP followed by MeIQx and MeIQ (Jahurul et al. 2010). Based on the Spanish National consumption database, dietary exposure to acrylamide from potato crisps (based on a 3-day food record) was 0.053 µg/kg body weight for the adult population (17–60 years) and 0.142 µg/kg body weight for children (7–12 years), similar to other European countries (Arribas-Lorenzo and Morales. 2009). CML exposure from a MRP-high diet was shown

to be 11.28 mg/day, while a MRP-low diet resulted in exposure of 5.36 mg/day in adolescents aged 11-14 years old (Delgado-Andrade et al. 2012). Dietary melanoidins represent the most abundant MRP in the human diet and ranges between 10-12 g per day for individuals consuming a typical western diet (Fogliano and Morales. 2011, Pastoriza and Rufian-Henares. 2014). For example, the estimation of dietary melanoidins from coffee ranged between 0.5 to 2.0 g per day. The intake of melanoidins in bread and dry biscuits ranged between 1.8-15 g and 3.2-8.5 g per day, respectively (Fogliano and Morales. 2011).

When foodstuffs undergo MR, the nutritional value of food is reduced, and some proteins are lost or become non-digestible, as reviewed by Tuohy et al. (2006). For example, exposing glucose and lysine to different heating periods caused loss of lysine (Adrian. 1974). Moreover, protein efficiency ratio (PER) decreases during MR. For example, the interaction between glycine and glucose reduced the PER by 22%, which reduced digestibility of nitrogen and metabolism of proteins measured in animals (Adrian. 1974). Increased amount of nitrogen in stool samples were also measured in young people who consumed a MRPs-rich diet (Seiquer et al. 2006). MRPs presence also affects trace element bioavailability. In an in-vitro study, the presence of MRPs in the diet (brown diet) reduced iron bioavailability (Mesias Garcia et al. 2009). MRPs decreased the digestion of magnesium in MRP-fed rats by 13%, compared to non-MRP-fed animals (Delgado-Andrade et al. 2007). Moreover, phosphorus bioavailability was linked to the consumption of a diet rich in MRPs (Delgado-Andrade et al. 2011). However, some reports indicate that melanoidins are likely to play a significant role in the binding of dietary metals; thereby, leading to antioxidant and antimicrobial properties (Morales et al. 2012). In particular, melanoidins that were prepared from glucose and glycine (GG) had a high chelating affinity towards copper (iron II) (32%),

compared to melanoidins obtained from lactose and lysine (LL) and lactose N-acetyllysine (LLa) (Borrelli et al. 2002).

### 1.4.2 Effect of Maillard Reaction Products on Health:

The major concern arising from the Maillard reaction is the formation of compounds that are not naturally present in foodstuff. Time, high temperature, and other parameters generate products that may have detrimental health effects, such as mutagenicity, carcinogenicity, cytotoxicity, and metabolic diseases, or beneficial impacts such as antioxidant, antimicrobial, and antihypertensive properties.

#### 1.4.2.1 *Toxicity and Carcinogenicity*:

The MRPs that have been reported to possess toxic and carcinogenetic properties are HMF, Acrylamide, HCAs, and AGEs (Tuohy et al. 2006). HMF is considered a toxicological compound because it can be converted into 5-sulphooxymethylfurfural (SMF) by sulfotransferase (SURH et al. 1994) and into 5-chloromethylfurfural (CMF) via allylic chlorination (Surh and Tannenbaum. 1994). Both compounds are known to be toxic and mutagenic. The highest daily exposure to dietary HMF was estimated as 8.57 mg HMF/day (Arribas-Lorenzo and Morales. 2010), and since the oral $LD_{50}$ was found to be 3.1 g/kg body weight in rats (Ulbricht et al. 1984), it can be considered that normal HMF intake may only represent a long term health risk. HMF was also shown to induce aberrant crypt foci of the colon in experimental animals (Archer et al. 1992). Skin papillomas caused by HMF have been reported in studies on rodents (Surh et al. 1994). Moreover, DNA damage, cytotoxicity of the kidney, and mutagenicity of the liver have been reported for HMF in mammalian cells (Schoental et al. 1971, Janzowski et al. 2000, Capuano and Fogliano.

2011, Lee et al. 1995). Specifically, HMF decreased the amount of glutathione, which is an important antioxidant that prevents damage to cellular components by reactive oxygen species (Lee et al. 1995).

Acrylamide was listed as a food-borne toxicant in 2002 by the Swedish National Food Administration, and it is considered a potentially carcinogenic and toxic compound (Tareke et al. 2002). As summarized in a review by Capuano and Fogliano. 2011, several studies demonstrated that acrylamide possesses cytotoxic, genotoxic, and tumorigenic activities. In a study using rodents, the exposure of acrylamide in different amounts led to an increase in the risk of developing cancer in the lung, thyroid, skin, and pancreas (Beland et al. 2013). Previous studies indicated that the metabolism of acrylamide further converted to N-acetyl-S-(3-amino-3-oxopropyl)-cysteine (AAMA), and the oxidation of AAMA into AAMA-sulfoxide induced kidney and bladder toxicity (Ramu et al. 1995, Capuano and Fogliano. 2011). However, the actual mechanisms responsible for dietary acrylamide carcinogenicity are still not well documented (Capuano and Fogliano. 2011, Tuohy et al. 2006).

Because heterocyclic amines (HCAs) are known as mutagenic and carcinogenic compounds, several studies indicated that red meat might be a risk factor for colorectal cancer (Cross and Sinha. 2004). HCAs are converted into genotoxic compounds by hepatic cytochrome P-450 1A2 enzyme (CYP1A2), which is activated by many factors, such as HCAs-rich diet. Specifically, CYP1A2 converted dietary HCAs into MeIQx and PhIP that are found in human urine (Boobis et al. 1994). In 1993, MeIQ, MeIQx, and PhIP were categorized as carcinogenic compounds by the International Agency for Research on Cancer, and IQ might also be a human carcinogen. PhIP, but not IQ, has been shown to induce colon tumors in rodents (Canzian et al.

1994). Moreover, liver tumors were induced in mice fed 0.06% of MeIQx that was extracted from foods (Ohgaki et al. 1987), and 0.03% of MeIQ that was isolated from broiled sardines induced tumors in various organs, such as the Zymbal gland, oral cavity, colon, skin, and mammary gland of the rat (Kato et al. 1989). Intestinal tumors were found in Nagase analbuminemic rats that were fed 0.04% to 0.01% of PhIP (Ochiai et al. 1991). Colonic aberrant crypt (AC) was found in the large intestine of rodents after 12 weeks of PhIP oral administration (Takahashi et al. 1991).

The potential role of endogenous AGEs and RAGE receptors in cancer risk has been extensively studied (Yamagishi et al. 2015). However, the pathological implications regarding the dietary AGEs and development of colorectal cancer risks have become more controversial. Elevated glyceraldehyde –AGEs levels were associated with the risk of rectal cancer, but were not linked to the risk of colon cancer based on 1,055 colorectal cancer cases (Kong et al. 2015). Increased risk of pancreatic cancer was found to correlate with dietary CML-AGE consumption, particularly in male pancreatic cancer patients (Jiao et al. 2015). In contrast, melanoidins, mainly from coffee, have generally been reported as potentially protective against cancer (Vitaglione et al. 2012, Gasscht et al. 2015, Ludwig et al. 2014). In *vitro* studies have shown significant anti-proliferative effects of melanoidins from heated potato fiber (Langner et al. 2013, Langner et al. 2011), miso and soy sauce (Kamei et al. 1997) and coffee (Vitaglione et al. 2012). However, because melanoidins are likely to behave similarly to fiber in the colonic microbial ecosystem, it has been suggested that most anti-cancer properties may derive from microbial fermentation metabolites (Ludwig et al. 2014, Jaquet et al. 2009).

1.4.2.2 *Metabolic and Cardiovascular Diseases:*

The more common emerging evidence of MRPs in the pathogenesis of metabolic and cardiovascular diseases are dietary AGEs through their binding with the receptor for advanced glycation end products (RAGEs) (Goldin et al. 2006,Grillo and Colombatto. 2008,Hartog et al. 2007). The binding of AGE-RAGE in the endothelial cells activates the transcription nuclear factor-kappa B (NF-κB), which induces pro-inflammatory cytokines and up regulates inflammation, notably in association with the development of diabetes and cardiac dysfunction (Hartog et al. 2007, Goldin et al. 2006). AGEs have been used as health biomarkers of several human diseases and conditions (Tessier and Birlouez-Aragon. 2012), such as inflammatory processes (Van Puyvelde et al. 2014), cardiovascular and metabolic diseases (Prasad et al. 2012,Yamagishi et al. 2017,de Vos et al. 2016) and aging (Wagner et al. 2016). Cai *et al* (2004) found that a high-AGE diet enhanced low-density lipoprotein (LDL), which induces vascular toxicity through protein kinase stimulants in diabetic patients. In addition to heart failure, dietary AGEs were shown to induce Type 1 diabetes in non-obese-diabetic mice (Peppa et al. 2003). A diet high in AGEs induced inflammatory mediators such as TNF-α, which contributes to the development of diabetes (Vlassara et al. 2003). In addition, a reduction in dietary AGE intake led to lower levels of circulating AGE and improved insulin sensitivity in db/db mice (Hofmann et al. 2002) and reduced possibly cardiovascular associated mortality in renal failure patients (Uribarri et al. 2003). AGEs were found to be involved in aging and in neurodegenerative pathways were reviewed by Grillo and Colombatto. (2008).

CML has been identified in tissues (Wang et al. 2012), plasma (Teerlink et al. 2004), urine, and feces (Delgado-Andrade et al. 2012). Although, CML is produced within the organism

endogenously (AHMED et al. 1986), several studies indicate that a significant correlation exists between dietary AGE content and CML serum in health people, as reviewed by Uribarri et al. (2005). A recent study carried out by Tessier *et al* (2016) found that the accumulation of dietary CML-fed mice was high in the kidney, intestine, and lungs, compared to native CML-fed mice. Serum levels of CML were found significantly higher in patients with diabetes, compared to healthy subjects (Jara et al. 2012). Pyrraline was found in the extracellular matrix of glomerular and arteriolar renal tissues from both diabetic and aged nondiabetic people (Monnier et al. 1992). The highest level of pentosidine was found in lens proteins of diabetic and uremic patients (Monnier et al. 1992).

1.4.2.3 *Antioxidant, Antimicrobial and Antihypertensive Activities*:

The beneficial effects of antioxidant properties of MRPs have been detected in some compounds, such as FL, HMF, and melanoidins. Amadori compounds might exert a moderate effect on the antioxidant activity of dehydrated onion and garlic during storage (Moreno et al. 2006). The pro-oxidant properties were observed in the early stages (FL) of pasta (Anese et al. 1999). Beside other wide range of products, HMF was found to play an important role in the antioxidant capacity of honey (Gheldof et al. 2002). Although the early and intermediate MRPs were shown to exert moderate antioxidant activity (Rufian-Henares and Delgado-Andrade. 2009), melanoidins are believed to be the major antioxidant MRPs (Rufian-Henares and Morales. 2007b).

Melanoidins are known as antioxidants, thus, several studies point out that food melanoidins could prevent gastrointestinal tract cancers (Morales et al. 2012). Melanoidins, extracted from different foods, such as roasted barley (malts) (Milic et al. 1975), cocoa (Hofmann. 1999), bread crust, and coffee (Fogliano and Morales. 2011), have been shown to enhance

antioxidant capacity (Somoza et al. 2005). For example, a significant increase of antioxidant activity was reported in the plasma of healthy people after an intake of 200 ml coffee (Natella et al. 2002). This result was in agreement with those reported by Vitaglione *et al* (2010), demonstrating a decrease in liver damage in rodents fed melanoidins extracted from coffee. In addition to coffee, malt and bread crust were found to increase the activity of chemopreventive enzymes of the kidney and liver and to decrease oxidative stress levels in the plasma of rodents (Somoza et al. 2005). The beneficial effects of MRPs on the antimicrobial and antihypertensive properties have been studied with melanoidins (Rufian-Henares and Morales. 2007a, Wang et al. 2011). Coffee melanoidins demonstrasted higher antimicrobial activities towards *Geobacillus stearothermophylus var. calidolactis* (Rufian-Henares and Morales. 2006). Melanoidin fractions were shown to suppress *Helicobacter pylori* infection in *vitro* and in *vivo* studies (Hiramoto et al. 2004). Moreover, water-soluble melanoidins were shown to possess antimicrobial properties towards pathogenic *E.coli* strains by disrupting their membranes (Rufian-Henares and Morales. 2008). Data from *in vitro* and *in vivo* studies indicated that melanoidins fractions from bread crust and coffee have a prebiotic activity similar to that of dietary fiber (Wang et al. 2011, Jaquet et al. 2009). For example, bread crust stimulated growth of beneficial bacteria, such as *Bifidobacterium* spp (Borrelli and Fogliano. 2005). The antihypertensive activity of melanoidins isolated from coffee and beer has been investigated only through *in vitro* ACE-inhibitory activity (Rufian-Henares and Morales. 2007b).

## 1.5 MRPs and Gut Microbiome and Metabolome

### 1.5.1 Human Gut Microbiome and Metabolome

In the last decade, the human microbiome/microbiota has received extreme attention from basic and medical scientists, and it is now well established that the human body hosts up to 100 trillion ($10^{14}$) microbes. The vast majority of them are located in the human gastrointestinal tract (GIT), which has become the most investigated microbial ecosystem (Ley et al. 2006). While microbiota composition is subject to strong individuality, the core human gut microbiota can be defined (Turnbaugh et al. 2009, Arumugam et al. 2011). The vast majority of colonic microorganisms depend on undigested dietary elements to support their metabolic needs, but some genera have also evolved to utilize other microbial by-products or host-derived compounds (Carbonero et al. 2012). The potential involvement of the gut microbiome has been extensively studied and reviewed for diseases, such as intestinal cancer (Candela et al. 2014,O'Keefe et al. 2015), inflammatory bowel diseases (Wehkamp and Frick. 2017), diabetes and metabolic syndrome, obesity (Delzenne et al. 2015,Kahn and Flier. 2000) and more recently brain diseases (Fung et al. 2017).

Studies revealed a high level of variability in microbiota due to dietary habits, including short and long term dietary habits that impact the gut microbiome (Ley et al. 2006). For example, it has been reported that long-term diets were associated with the type of enterotypes of gut microbiota, but short-term diets were correlated with gut microbiota composition (Wu et al. 2011). Wu *et al* (2011) found that the prevalence of *Bacteroides* enterotype was strongly associated with the consumption of animal protein and saturated fats, but the dominance of the *Prevotella* enterotype was linked to a carbohydrate-based diet. Consequently, the interaction between diet and

the gut microbiome has been involved in both etiology and preservation from diseases (Louis et al. 2007, O'Keefe. 2016, O'Keefe. 2008).

Gut bacteria degrade undigested foods by two main metabolic pathways: saccharolytic and proteolytic. On the one hand, saccharolytic bacterial species, such as *Bacteroides* spp, *Bifidobacterium* spp, *Ruminococus* spp, *Peptostreptococcus* spp and *Roseburia intestinalis* hydrolyze non-digestable carbohydrates into monomeric sugars that convert to beneficial products, such as short-chain fatty acids (SCFAs), principally acetate, propionate, and butyrate (Gibson and Roberfroid. 1995,Duncan et al. 2002). On the other hand, microbial metabolism of proteins, such as *Bacteroides* spp, *Propionbacterium* spp, *Eubacterium* spp, and *Peptococcus* spp degrade peptide and amino acids into a variety of products including short or branched-chain fatty acids, and other metabolite compounds, some of which are potentially toxic, such as uremic toxins (Evenepoel et al. 2009, Macfarlane et al. 1986), phenols and amines. While metabolites from these two pathways are arguably dominant in terms of abundance, the complete metabolome comprises at least tens of thousands of different molecules (42,003 in the most recent version of the Human Metabolome Database) (Wishart et al. 2016). Since MRPs are molecules with both carbohydrate and proteic structures, it is likely that there are less bacterial members able to degrade them, and that microbial consortia are probably needed to fully metabolize them to end-products.

### 1.5.2 Known Microbial Interactions between Microbes and MRPs

#### 1.5.2.1 *Impact of MRPs on Food-Associated Microbes*

The impact of MRPs and associated environmental parameters on microorganisms has been studied mostly by culture-dependent studies, as reviewed in (Helou et al. 2014). The first

study was by Hachisuka (1955), describing the impact of heat treatments on germination spores of bacteria. The germination time of *Bacillus subtilis* spores decreased after exposing media to heat treatments (Hachisuka et al. 1955). This finding was in agreement with the study reported by Viswanathan and Sarma (1957), describing an inhibitory growth of *Lactobacillus bulgaricus* in heated milk powders. On the contrary, Foster observed the growth of lactic acid bacteria in heated milk (Foster. 1952). Lately, some studies have attempted to shed light on the effect of MRPs on microorganisms. For instance, Stecchini *et al* (1991) found that MRPs inhibited the growth of food-poisoning microorganism, such as *Staphylococcus aureus*, *Salmonella* Typhimurium, and *Salmonella* Enteritidis. Several studies indicated microorganisms that were isolated from different environments were able to degrade and use MRPs from different stages as shown in (Table 1.2).

FL was shown to be preferentially used as a carbon source by *Salmonella* Typhimurium in batch cultures, compared to AGEs and melanoidins (Chalova et al. 2012). Moreover, *Escherichia coli* were found to use FL as an energey substrate. *Escherichia coli* has fructoselysine-6-phosphate deglycase enzyme that catalyzes the ATP-dependent phosphorylation of fructoselysine to fructoselysine 6-phosphate, and subsequently to lysine and glucose 6-phosphate. Thus, this enzyme reached high activity levels during fructoselysine utilization (Wiame et al. 2002). Another study identified glucoselysine-6-phosphate deglycase produced by *Enterococcus faecium* to convert fructoselysine into lysine and glucose 6-phosphate, which was used as an energy source (Wiame et al. 2005).

Among intermediate MRPs, it was found that SMF and CMF, which are derived from HMF, had direct mutagenicity towards *Salmonella* Typhimurium (Sommer et al. 2003). In addition to HMF, the formation of acrylamide during the deep-frying of French fries can be effectively

lowered by prior lactic acid fermentation carried out by *Lactobacillus plantarum* (Baardseth et al. 2006). Moreover, a recent study described the reduction of acrylamide formation in wheat biscuits by lactic acid bacteria fermentation, including *Lactobacillus sakei, Pediococcus pentosaceus* and *Pediococcus acidilactici* (Bartkiene et al. 2016). In a recent review, several studies indicated that acrylamide was catalyzed to ammonia and acrylic acid by some microorganisms, which produce amidases (an enzyme found in some microbes) (Duda-Chodak et al. 2016). In addition to above, data from microorganism studies found that some gram- positive and gram- negative bacteria could detoxify HCAs by binding of HCAs to the peptidoglycan layer and the outer membrane of microbes under physiologically conditions, which have been reviewed in details by Knasmuller et al (2001).

In the advanced products of MR, the reduction of AGEs and melanoidins levels were 37% and 15%, respectively, after incubating with *Salmonella* Typhimurium in batch cultures (Chalova et al. 2012). Inhibition of microbial growth by MRPs has been studied (Einarsson et al. 1983). High molecular weight MRPs inhibited the growth of *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus*, compared to low molecular weight MRPs (Einarsson et al. 1983). These results are in agreement with studies by Rufian-Henares and Morales (2006), demonstrating higher antimicrobial activity was found in high molecular weight of melanoidins, such as coffee. This approach was successfully tested with darker coffee that exhibited high antimicrobial activity against *E.coli*, and reported that melanoidins can damage both inner and outer membranes of the pathogenic bacteria strain (*E.coli*) (Rufian-Henares and Morales. 2008). Other studies showed that the antimicrobial activity of melanoidins were higher towards gram-positive microorganisms compared to gram-negative microbes (Rufian-Henares and Morales. 2006, Rufian-Henares and Morales. 2007a,b).

1.5.2.2 *Known Microbial Metabolites of MRPs*:

1.5.2.2.1 Metabolite of Amadori Products:

The available data for metabolism of early MRPs found that the urinary excretion of ingested fructolysine was 60-80% in rats and 3-10% in humans (Faist and Erbersdobler. 2001). It has been reported that the intestinal absorption rate of Ɛ-fructoselysine was higher than α-fructoselysine (Erbersdobler et al. 1981). Another study found that excretion of NƐFructoselysine in urine and feces was very low 3.68% in humans and 11.2%, in rats (Erbersdobler and Faist. 2001). Thus, several studies indicated that the remainder of $N^{\varepsilon}$Fructoselysine was more likely to be degraded by intestinal microorganisms or accumulate in different tissues, according to a review (Faist and Erbersdobler. 2001).

1.5.2.2.2 Metabolite of Advance MRPs (Pre-Melanoidins):

HMF is converted to 5-hydroxymethyl-2-furoic acid (HMFA), during its metabolism and is excreted through urine in mammals (Godfrey et al. 1999, Husoy et al. 2008). Acrylamide is converted into other substances, such as glycidamide or conjugated with glutathione (GSH). Both glycidamide and GSH are further converted into N-acetyl-S-(3-amino-3-oxopropyl)-cysteine (AAMA) and other substances that are excreted with urine (Boettcher et al. 2006). The excretion of CML in feces was high for rich-MRP (3.52 mg/day), compared to low-MRP (1.23 mg/day). However, the elimination of CML in urine was not significantly different between high and low MRPs (Delgado-Andrade et al. 2012). The large amounts of dietary CML recovered in urine (accounted for 26–29%) and in feces (accounted for 15–22%), but more than 50% of CML was not yet accounted for, which might be degraded by the intestinal microbiota (Ames. 1990).

1.5.2.2.3 Metabolites of Melanoidins:

The urinary excretion rate of melanoidins depends on molecular weight. To clarify, the rate of excretion of high molecular weight (HMW) melanoidins was 4.3%, compared to low molecular weight (LMW), which was 27% (Finot and Magnenat. 1981). Importantly, several studies indicated that major dietary sources of melanoidins remain in the gastrointestinal tract where they exhibit biological action, according to a review by Tagliazucchi and Bellesis (2015).

1.5.2.3 *The Limited Knowledge on the Impact of MRPs on Gut Microbiota*

Most research focused on impact of dietary MRPs using *in vitro* assays using fermentation with human fecal samples or *in vivo* models by means of animal studies. In the early observation of the effect of MRPs on the gut microorganism *in vitro* study, Jemmali (1969) observed increases in the growth rates of three *Lactobacilli* strains, but no effect on *E.coli* growth in batch cultures of MRPs (Jemmali. 1969). Moreover, Horikoshi *et al* (1981) detected the impact of browning products, prepared from D-glucose and glycine, on the growth both aerobic and anaerobic *Lactobacilli* in the microflora of rats. From the small number of in vitro studies, it appears that MRPs stages influence the response of gut microbiota members (Table 1.3).

As stated previously, excretion of $N^{\varepsilon}$Fructoselysine (FL) in urine and feces is very low (Erbersdobler and Faist. 2001), and it has been shown that the human colonic microbiota can degrade FL after 4 hours of anaerobic incubation with human fecal samples (Hellwig et al. 2015). Moreover, gut bacteria related to *Intestinimonas AF211* were found to contain genes coding for a butyrate-acetoacetic-CoA transferase that can convert Amadori products into butyrate in the human intestine (Bui et al. 2015). A negative correlation between the fecal of *Bifidobacteria* counts and

Amadori product was found in a study using human fecal samples, but no correlations were discovered between cecal *Bifidobacteria* numbers of rats and Amadori product (Seiquer et al. 2014).

Fecal suspensions of $N^{\varepsilon}$Carboxymethyllsine (CML) and pyrraline (PYR), the type of AGEs produced from the intermediate stage, were degraded by human gut microbiota after 24 hours (Hellwig et al. 2015). However, CML did not impact the growth rates of three strains of *E.coli* that were isolated from human and piglet feces, and there was no degradation of CML observed in the presence of *E.coli* (Helou et al. 2014). The number of *Lactobacilli* and CML intake correlated negatively for both human and animal studies (Seiquer et al. 2014). In addition to CML, negative correlations were found between Hydroxymethylfurfural (HMF) intake and *Lactobacilli*, *Escherichia*, and *Shigella* counts both *in vivo* (animal) and *in vitro* (human) studies (Seiquer et al. 2014). Moreover, HMF was converted into furfural alcohol, which is less toxic after it was incubated with enteric bacteria, such as *Klebsiella*, *Enterobacter*, and *Escherichia* in short time incubations. According to authors, these biotransformations might be valuable in the detoxification of furfural compounds (Boopathy et al. 1993). IQ, a known HCA as mutagenic compound, was converted into innocuous metabolite structures after incubation with human fecal samples (Bashir et al. 1987). However, activation of IQ by *Eubacterium* and *Clostridium* into potentially mutagenic 7-hdroxy "IQ" compounds has also been shown in *Salmonella* (Vantassell et al. 1990).

Data from animal studies show that melanoidins escape digestion and pass into the lower gastrointestinal tract (Finot and Magnenat. 1981). Subsequently, they are likely to be degraded by gut microorganisms (Faist and Erbersdobler. 2001). Indeed, melanoidins have been shown to have potential prebiotic activity (Wang et al. 2011). For instance, an increase in the number of gut

bacteria was observed during fermentation with bread melanoidins, which could be used as sources of carbon and nitrogen, particularly *Bifidobacteria* strains, which had the highest growth among anaerobic bacteria (Borrelli and Fogliano. 2005). Moreover, because melanoidin fractions were found in coffee (Daglia et al. 2008), melanoidins might increase the proportion of *Bacteroides-prevotella*, compared to total cell counts after healthy human fecal samples were incubated with different roasted coffee (Reichardt et al. 2009). According to their findings, the composition of human fecal microbiota was changed during incubation with coffee. This result was in accordance with those studied by Jaquet *et al* (2009), showing increase in *Bifidobacterium* spp and metabolic activity in healthy people after a three week test period of the consumption of coffee.

**1.6 Conclusions and Perspectives:**

The chemistry of dietary MRPs is relatively well known, but the biological impact of these molecules is less understood. While acrylamide, HCA and HMF have relatively well established detrimental health properties, the normal dietary exposure to these MRPs is arguably low, even in the case of the Western diet. More *in vitro* or animal studies using relevant concentrations of MRPs, or actual MRP-rich food products are needed in order to better assess the status of MRPs towards different health conditions and diseases.

A significant fraction of dietary MRPs are large molecules that mostly escape digestion, but little is known about their fate in the gastrointestinal tract and their interaction with microbiota. *In vitro* models have been used most commonly to study the impact of MRPs on gut microbiota. However, it can be argued that *in vitro* models would actually be better suited to determine the metabolite profiles resulting from microbiota fermentation of MRPs. *In vivo* studies are greatly needed to track the impact of MRPs on gut microbiota as well as biomarkers of health.

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**Table 1.1:** Examples of MRPs content of commonly consumed foods:

| MRP type | Food item | Average MRP concentration | References |
|---|---|---|---|
| $N^{\varepsilon}$Fructoselysine (FL) | Soybean | 66.55µg/g | (Zilic et al. 2014) |
| | Fresh Pasta | 16-18.8 mg/100 g of protein | (Garcia-Banos et al. 2004) |
| | Dried Pasta | 44.4-462 mg/100 g of protein | |
| 5-Hydroxymethylfurfural (HMF) | Soluble Coffee | 110 mg/kg | (Arribas-Lorenzo and Morales. 2010) |
| | Cookies (sucrose) | 19 µg/g for 10 min at 230C° | (Gokmen et al. 2007) |
| | Cookies (glucose) | 38 µg/g for 10 min at 230C° | |
| Acrylamide | Potato Chips | 628 µg/kg | (Capuano and Fogliano. 2011) |
| | French Fries | 350 µg/kg | |
| | Biscuits | 317 µg/kg | |
| Heterocyclic amines (HCAs) | Fried Bacon | 17 ng/g | (Puangsombat et al. 2012) |
| | Fried Tilapia | 16.29 ng/g | |
| | Fried Pork | 13.91 ng/g | |
| | Fried Beef | 8.92 ng/g | |
| Advanced Glycation End products (AGEs) | Butter | 100 KU/g | (Goldberg et al. 2004) |
| | Mayonnaise (fats) | 265 KU/g | (Van Nguyen. 2006) |
| | American Cheese (proteins) | 87 KU/g | |
| | Pancakes (carbohydrates) | 10 KU/g | |

**Table 1.1 (Cont.)**

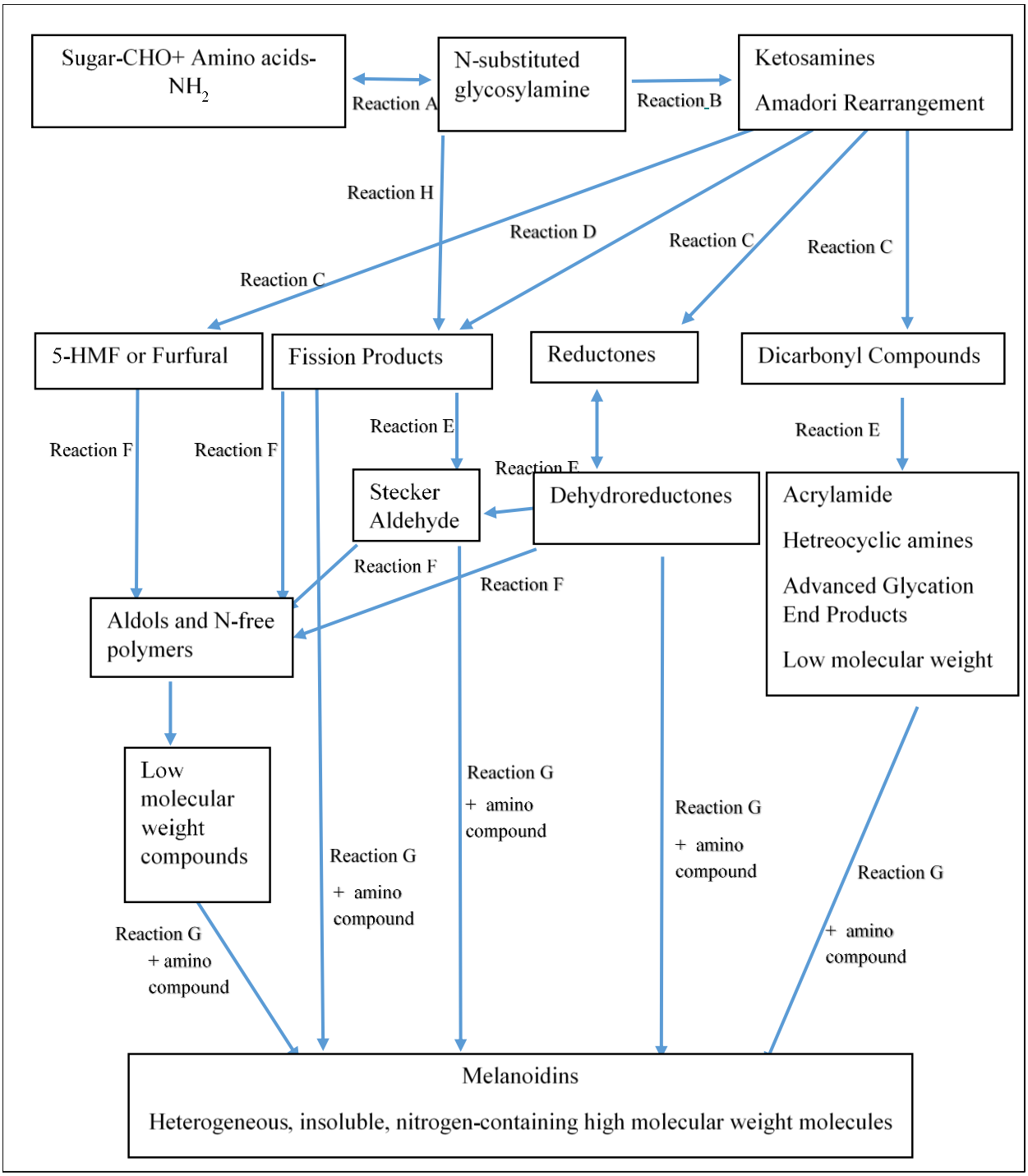
| | | | |
|---|---|---|---|
| $N^{\varepsilon}$Carboxymethyllsine (CML) | Sterilized Milk | 2066 nM of protein | (Ahmed et al. 2005) |
| | Pasteurized Milk | 887 nM of protein | |
| | Raw Milk | 337 nM of protein | |
| | White Bread Crust | 382 mg/kg of protein | (Assar et al. 2009) |
| | Wholemeal Bread Crust | 329 mg/kg of protein | |
| | Doner Kebab | 2357.87 mg/kg of protein | (Hull et al. 2012) |
| Melanoidins | Sourdough Loaves | 30 g/100g of crust | (Fogliano and Morales. 2011) |
| | Soluble Coffee | 22 g/100 g | |
| | Roasted Barley | 4.15% of 0.7 to1.0 kg | (Milic et al. 1975) |

**Table 1.2:** Previous reports on the impact of MRPs on microorganisms:

| MRP type | Microorganisms | The result of Study | References |
|---|---|---|---|
| FL | *Salmonella Typhimurium* | Utilization 95% of FL as carbon and energy sources | (Chalova et al. 2012) |
| | *E.coli* | Conversion FL into lysine and glucose 6 phosphate | (Wiame et al. 2002) |
| | *Enterococcus faecium* | Conversion FL into lysine and glucose 6 phosphate | |
| HMF (SMF)(CMF) | *Salmonella Typhimurium* | Mutagenicity in *Salmonella Typhimurium* | (Sommer et al. 2003) |
| Acrylamide | *Lactobacillus plantarum* | Reducing the levels of acrylamide | (Baardseth et al. 2006) |
| | *Lactobacillus sakei*, *Pediococcus pentosaceus* and *P. acidilactici* | Reducing the levels of acrylamide | (Bartkiene et al. 2016) |
| AGEs | *Salmonella Typhimurium* | Utilization 37% of AGEs as carbon and energy sources | (Chalova et al. 2012) |
| Melanoidins | *Salmonella Typhimurium* | Utilization 15% of Melanoidins as carbon and energy sources | (Chalova et al. 2012) |
| | *E.coli* | Inhibition the growth rates | (Rufian-Henares and Morales. 2008) |

**Table 1.3:** Current available data of the effect of MRPs on colonic microbiota:

| The type of MRPs | The type of Gut bacteria | The results of study | References |
|---|---|---|---|
| FL | Viable microbiota (stability) | Use as carbon source after 4 hours | (Hellwig et al. 2015) |
| | *Intestinimonas AF211* | Conversion FL into butyrate | (Bui et al. 2015) |
| | *Bifidobacteria* counts | Decrease growth rates | (Seiquer et al. 2014) |
| CML/PYR | Viable microbiota (stability) | Use as carbon source for 24 hours | (Hellwig et al. 2015) |
| CML | *E.coli* | No effect on growth rates | (Helou et al. 2014) |
| CML | *Lactobacilli* counts | Decrease growth rates | (Seiquer et al. 2014) |
| HMF | *lactobacilli*, *Escherichia*, and *Shigella* counts | Decrease growth rates | (Seiquer et al. 2014) |
| HMF | *Klebsiella*, *Enterobacter*, and *Escherichia* | Conversion HMF into furfural alcohol | (Boopathy et al. 1993) |
| HCAs (IQ) | Gut microbiota | Conversion IQ into innocuous metabolites | (Bashir et al. 1987) |
| IQ | *Eubacterium* and *Clostridium* | Conversion IQ into7-hdroxy | (Vantassell et al. 1990) |
| Melanoidins | *Bifidobacteria* | Use as carbon source and increase growth rates | (Borrelli and Fogliano. 2005) |
| Melanoidins | *Bacteroides-prevotella* | Increase growth rates | (Reichardt et al. 2009) |


Sugar-CHO+ Amino acids-NH2
Reaction A
N-substituted glycosylamine
Reaction B
Ketosamines
Amadori Rearrangement
Reaction H
Reaction D
Reaction C
Reaction C
Reaction C
5-HMF or Furfural
Fission Products
Reductones
Dicarbonyl Compounds
Reaction F
Reaction F
Reaction E
Reaction E
Reaction E
Stecker Aldehyde
Dehydroreductones
Acrylamide
Hetreocyclic amines
Advanced Glycation End Products
Low molecular weight
Reaction F
Reaction F
Aldols and N-free polymers
Low molecular weight compounds
Reaction G + amino compound
Reaction G + amino compound
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Reaction G + amino compound
Melanoidins
Heterogeneous, insoluble, nitrogen-containing high molecular weight molecules

**Figure 1.1**

**Figure 1.1**: Maillard Reaction Products stages (adapted from the initial description by Hodge in 1953 to reflect current knowledge). **1. Early stage:** condensation of the carbonyl group of reducing sugar with the amino group of amino acids (Reaction A), resulting in N-substituted glycosylamine and water. **2. Intermediate stages:** Conversion of glycosylamine through Amadori rearrangement to form ketosamines (Reaction B) and other products. Amadori products are dehydrated and hydrolyzed to form 5-Hydroxymethylfurfural (HMF) (Reaction C), which gives rise to either Aldose or N-free polymers (Reaction F). Reductones can be formed from either dehydration of sugars or Amadori product (Reaction C) leading to Aldose and N-free polymers (Reaction F) or Stecker Aldehydes (Reaction E). Stecker aldehydes are formed by fragmentation of amino acids, which enter browning reactions either by the aldehyde formed that can take part in aldol condensation which forms nitrogen-free polymers (Reaction F). Amadori product and N-substituted glycosylamines can be fragmented fission products (Reaction D and H). In addition, fragments of MRPs produce reactive dicarbonyl compounds that can act as precursors of acrylamide, heterocyclic amines, advanced glycation end products (AGEs), and low molecular weight compounds (Reaction C). **3. Advanced stages:** Melanoidins include a wide array of heterogeneous colored molecules Dehydroreductones, fission and dicarbonyl compounds, furfural and Aldose react with amino acids (Reaction G) to form melanoidins.

CHAPTER TWO

RESEARCH ARTICLE

# Repeated oral exposure to $N^{\varepsilon}$-Carboxymethyllysine, a Maillard Reaction Product, alleviates gut microbiota dysbiosis in colitic mice

Nesreen ALJahdali MS[1], Pascale Gadonna-Widehem PhD[2], Carine Delayre-Orthez PhD[2], David Marier MS[2], Benjamin Garnier MS[2], Franck Carbonero PhD [1,3*] and Pauline M Anton PhD[2].

[1]Cell and Molecular Biology Program; University of Arkansas, AR, USA

[2]Expression des Gènes et régulation Epigénétique par l'Aliment UP 2012.10.101. ; Institut Polytechnique UniLaSalle, Beauvais, France

[3]Department of Food Science; University of Arkansas, AR, USA

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**2.1 Abstract:**

**Background:** Diet is suggested to participate in the etiology of Inflammatory Bowel Diseases (IBD). Repeated exposure to Maillard reaction products (MRPs), molecules resulting from reduction reactions between amino acids and sugars during food heating, have been reported to be either potentially detrimental or beneficial to health.

**Aims:** the aim of this study is to determine the effect of repeated oral ingestion of $N^{\varepsilon}$Carboxymethyllysine, an advanced MRP, on the onset of two models of experimental IBD and on the composition of the gut microbiota of mice.

**Methods:** Mice received either saline (control) or $N^{\varepsilon}$Carboxymethyllysine daily for 21 days. For the last week of treatment, each group was split into sub-groups, receiving Dextran sulfate sodium salt (DSS) or Trinitrobenzene sulfonic acid (TNBS) to induce colitis. The intensity of the inflammation was quantified and cecal microbiota was characterized by bacterial 16S rRNA amplicon sequencing.

**Results:** Daily oral administration of $N^{\varepsilon}$Carboxymethyllysine did not induce intestinal inflammation and had limited impact on the gut microbiota composition (Bacteroidaceae increase, Lachnospiraceae decrease). DSS and TNBS administration resulted in an expected moderate experimental colitis with a shift of Bacteroidetes/Firmicutes ratio and a significant Proteobacteria increase, but with distinct profiles: different Proteobacteria taxa for DSS, mainly Enterobacteriaceae for TNBS. While $N^{\varepsilon}$Carboxymethyllysine exposure failed to prevent the inflammatory response, it allowed maintenance of healthy gut microbiota profiles in DSS (but not TNBS) treated mice.

**Conclusions:** Repeated oral exposure to CML limits the dysbiosis in experimental colitis. IBD patients may modulate their microbiota profile by regulating the level and type of dietary MRP consumption.

**Keywords:** Maillard Reaction Products - $N^{\varepsilon}$-Carboxymethyllysine – Intestinal microbiome – Inflammation – Dextran Sulfate Sodium salt colitis – TriNitroBenzene Sulfonic acid colitis

## 2.2 Introduction:

Gut microbiota has been the focus of the exponentially increasing numbers of studies because of its purported role in a variety of diseases [1-3]. Gut microbiota (or microbiome) is the complex community of microorganisms, such as Archaea, protozoans, fungi, and for the vast majority of bacteria that inhabit the gastrointestinal tract (GIT) of humans and other animals, which mainly ferment and degrade undigested dietary compounds [3, 4]. Arguably dietary habits are considered to be one of the main factors contributing to the diversity and shaping of human gut microbiota, since there is growing evidence that human associated microbes have been lost through the transition from ancestral to Western diet [5-7]. Inflammatory Bowel Diseases (IBD) and colon cancer are well known to be epidemiologically associated with Western diet consumption [5, 6], and gut microbiota dysbiosis (imbalanced composition) has been shown to be a potential contributor to their associated inflammatory process [8, 9]. Gut microbiota dysbiosis has also been shown in different animal models of IBD, including Trinitrobenzene sulfonic acid (TNBS) and Dextran sulfate sodium salt (DSS)-induced colitis in mice [10-12]. These two models have been widely used to study intestinal inflammation since they induce different immune, mucosal and microbiota alterations that reflect the clinical signs observed in IBD patients. While literature depicts links between diet composition and the intestinal microbiome [13], there are few data supporting the potential links between the mode of food preparation, such as food heating and chronic gut inflammation.

Heat processing of food generates several neoformed compounds among which Maillard reaction products (MRPs), a group of several compounds that influence the color, flavor, texture and nutritional value of food, are suspected to have health implications [14]. The intensity of such heating determines the types of MRPs present in food, being classified as initial (Amadori

Compounds), intermediate (i.e $N^{\varepsilon}$-Carboxymethyllysine, acrylamide, etc.) and final stage (melanoidins, etc.) [15]. MRPs are also produced in living bodies. To avoid any confusion, food intermediate compounds are named dietary advanced glycation end products (dietary AGEs) to discriminate them from endogenous AGEs. It has been established that Western diet, richer in lipids and proteins, is a significant source of dietary AGEs known to be a chronic risk for cardiovascular and kidney damage [16]. Among the two types of AGEs, $N^{\varepsilon}$-Carboxymethyllysine (CML) – a product of both lipoxidation and glycoxidation [17] –is considered to be the most important biomarker of MRP commonly measured in human blood and urine. CML levels are also used to infer the amount of AGEs consumed by individuals [18, 19]. Endogenous CML has been reported to be a reliable biomarker of oxidative stress and of long-term damage to proteins [20]. In some studies, CML has been associated with various diabetes complications[21-24] and age-related health conditions [25-27] while, in others, CML has been reported to be not correlated or negatively correlated with inflammation [28-30]. Dietary CML, present in more than 200 food items [16, 31], can be produced either by Amadori rearrangement products or by Amadori compound degradation, such as Fructoselysine (FL) that further reacts with proteins to form irreversible and highly stable compounds [31]. Since circulating CML is known to derive either from endogenous Maillard reaction, or CML present in food, or of conversion of AGEs by the gut microbiota [32, 33], the potential contribution of dietary CML on the onset of the inflammatory process has been hard to determine.

As MRPs may be largely present in the Western diet, they may be considered as one of the many dietary elements potentially incurring detrimental impact on human health. However, their fate in the digestive tract, specifically through the gut microbiota has been sparsely studied, and mainly *in vitro* [34-36]. The gut microbiota was shown to degrade MRPs, such as CML, pyrraline

(PYR) and maltosine (MAL) after anaerobic batch incubations with fecal suspension from healthy humans and they used them as a source of energy, carbon, and nitrogen [34]. Using quantitative polymerase chain reaction (qPCR) analysis, no differences were found in the total bacteria of adolescents who consumed white diet (low MRPs) versus brown diet (high MRPs), but a significant decrease was shown when rats consumed similar diets [37]. Recently, we have determined that ingestion of highly heated food protected mice against an experimental colitis and dysbiosis [38]. This food contained higher levels of dietary AGEs (such as CML), but also higher levels of final stages MRPs (i.e., melanoidins). In summary, all these studies relied on culture-dependent or targeted quantitative PCR approaches, but none were aimed at enabling an extensive and specific characterization of CML (or other MRPs) impact on the gut microbiota.

In order to gain a better understanding, this study aimed to determine the effect of repeated daily exposure to CML alone not only on the onset of the colonic inflammatory reaction, but also on the expected intestinal dysbiosis observed following both TNBS and DSS-induced experimental colitis in mice.

**2.3 Materials and Methods:**

**2.3.1 Chemicals:**

$N^{\varepsilon}$-Carboxymethyllysine (CML) was obtained from Polypeptide laboratories in Sigma-Aldrich in France. Dextran sulfate sodium salt (DSS) was purchased from MP Biomedicals while Trinitrobenzene sulfonic acid (TNBS) was purchased from Sigma-Aldrich in France.

**2.3.2 Animals and Study Design:**

Fifty male Balb/c mice 7 weeks old (18-20 g) were housed in stainless steel cages under a controlled temperature (21 ± 1°C) and a 12H light-dark cycle, with free access to food and water. Experiments were conducted at the Animal House Unit of LaSalle Beauvais after receiving the

prior approval of both the animal protocol review committee and the Picardy Counsil of Veterinary Office in France (C60-200-01). Mice were split in two groups and orally received CML (1.6 mg/kg/d *po* – 50 µl/mouse/d) or saline (50 µl/mouse/d) orally every day for 3 weeks. The last week of the experiments, each group was divided into three subgroups: two (n=9/subgroup) submitted to either Dextran Sulfate Sodium (DSS) or TriNitroBenzene Sulfonic acid (TNBS) colitis, and one serving as a control (n=7/subgroup).

At the end of the experiment, animals were sacrificed by cervical dislocation under general anesthesia (ketamine/xylazine 100 mg/kg i.p. 50% v/v). A midline laparotomy was then realized, macroscopic lesions were assessed and then pieces of colon were immediately removed and snap frozen until further analysis. To perform the analysis of the incidence of each treatment on the microbiota profile, caeca were ligated at both the ileo-caecal and the caeco-colonic junctions before their removal, and their contents were immediately collected under anaerobic conditions and snap frozen until further determination.

**2.3.3 Experimental Colitis**

*Colitis models:* In order to confirm that the effects of CML on colonic inflammation were not model-dependent, colitis was induced using two models widely used in the literature. The TNBS solution was diluted in 50% ethanol (100mg/kg, 50 µL IR) before being injected intrarectally (IR) via lubricated silicone tubing (4cm from the anus) under general anesthesia (ketamine/xylazine 100 mg/kg, 50% v/v, 1mL/kg ip). Animals were then kept head-down on a heating pad until full recovery from the anesthesia [39].

DSS (36-42000 Da) was solubilized in the drinking water (3% w/v) and was administered for five days. Bottles were changed every other day. For the last 2 days of the experiment, DSS was removed from the bottles [40].

*Macroscopic lesions:* The level of macroscopic lesions was represented as macroscopic damage scores (MDS) and expressed in arbitrary units (AU) according to the literature. Briefly, DSS lesions severity was evaluated based on weight loss (from 0 non loss – to 4 more than 20% loss), stool consistency (from 0 consistent to 4 diarrhoea), occult or gross bleeding (from 0 none to 4 gross bleeding) and gross blood content (from 0 non blood to 3 blood in more than 2/3 of the colon) [40, 41]. TNBS-induced lesions were evaluated based on the level of ulceration (from 0 normal appearance to 5 inflammation on more than 1cm), presence of adhesions (from 0 none to 2 major) and diarrhea (0 no – 1 yes) [39].

*Myeloperoxidase Activity:* The inflammatory reaction is a process associated with immune cell activation and neutrophil infiltration. Myeloperoxidase (MPO) is exclusively found in neutrophil cytoplasmic granules, and thus considered a reliable marker for quantifying the level of inflammatory reaction [42]. MPO activity assay was first described by Bradley et al [43]. MPO was extracted from colonic tissues collected at sacrifice as previously described [44].

Briefly, tissue samples were lysed in a phosphate buffer (50mM, pH=6) containing hexadecyl trimethyl ammonium bromide (0.5% m/v) with a polytron (PT 1200E, Kinematica AF, Switzerland). Homogenates were then submitted to 3 cycles of freezing and thawing (-80°C – 10 min / 37°C for 15 min), and then sonicated (Bioblock scientific, France) before centrifugation (6000 g – 4°C – 15 min). Supernatants were collected to measure MPO activity and protein concentration. MPO activity was assessed on supernatants diluted on a reaction buffer containing O-dianisidine dihydro-chloride (1mg/mL) and hydrogen peroxide ($5 \times 10^{-4}$ % v/v); purified MPO (1 U/mL) from human leucocytes (Sigma Aldrich, France) as a standard. Absorbance was measured at 450 nm after 15 minutes of incubation at room temperature. Supernatants total protein content was assessed based on Lowry method following the manufacturer's recommendations (Bio

Rad DC Protein Assay, France). The results were expressed as MPO units/mg protein where 1 unit of MPO is the amount of peroxidase necessary for transforming 1 µmol of hydrogen peroxide in 1 min at 25°C.

### 2.3.4 DNA Extraction and Amplicon Sequencing Strategy

Genomic DNA was extracted from cecal samples. Briefly, 100 mg cecal content and 1 mL of ASL buffer (Qiagen, Courtaboeuf, France) were pooled in 2 mL Lysing Matrix E tubes (MP Biomedicals, Santa Ana, USA). After mixing for 1 min with a vortex, the tubes were heated at 95°C for 5 min in a Thermomixer (Eppendorf, Montesson, France) then cooled in ice for 5 min. The tubes were transferred into the FastPrep® (MP Biomedicals, Santa Ana, USA) bead-beater for 2 runs of 1 min each at 6 m/s. After centrifugation (16000g at 4℃ for 1 min), the total DNA was extracted from the obtained lysates using a commercial kit (QIAamp DNA stool Mini Kit) following the manufacturer's protocol [42].

Illumina (San Diego, USA) MiSeq sequencing was used to investigate the gut microbiota by targeting the V4 region of the bacterial 16S rRNA gene of each group following the dual-indexed strategy developed by Kozich et al [45]. PCR amplifications were performed in 25 µL reactions with 22 µL of KAPA Hifi master mix (KAPA Biosystems, Wilmington, USA), 1 µL of DNA template and 2 µL of index primers. PCR was performed with initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 98 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min, with final extension at 72 °C for 7 min. PCR amplicons were examined on agarose gel electrophoresis by using 6 µL of ethidium bromide fluorescent dye to confirm the successful PCR.

### 2.3.5 Library Preparation and Quality Control:

Normalization of PCR products was performed with a SequalPrep kit (Invitrogen) following the manufacturer's instructions. Normalized amplicons were pooled and checked on agarose gels. Gel extraction of the correct amplicon was achieved by running agarose gel electrophoresis on 100 μL DNA, then extracting DNA from excised bands using the Min Elute Gel Extraction kit (Qiagen, Hilden, Germany). Library quality checks were performed by means of real-time q-PCR (RT-q-PCR) and TapeStation (Agilent®, Santa Clara, USA) to determine the concentration and size of DNA libraries, respectively. Q-PCR was performed with the KAPA Library Quantification Kit (KAPA Biosystems) following the manufacturer's recommendations. The q-PCR plate was placed in a thermocycler for initial activation at 95 °C for 5 min followed by 35 cycles. Amplicon size was determined again after gel extraction on the TapeStation (378 bp and 372 bp).

### 2.3.6 Bioinformatics and Statistical Analyses:

Data relating to inflammation are expressed as mean ±stand error of the mean (SEM). They were analyzed using Graph Pad Prism Software. Macroscopic lesion scores were compared using Kruskal-Wallis nonparametric test followed by Dunn's multiple comparisons post hoc test. All other data were submitted to analysis of variance (ANOVA) followed by Tukey post hoc test. *P* value <0.05 was considered to be significant.

All sequences were analyzed by using the Quantitative Insight into Microbial Ecology (QIIME) software package following the script in Ref. [46], which performs microbial community analysis. The results of microbiota counts were analyzed by Kruskal-Wallis test using past3 software. *P* value of <0.05 was considered to be significant between each group. Microbiota data were visualized by Non-Metric Multidimensional Scaling (NMDS) using the Bray-Curtis

similarity index. The significance of clustering by subgroups was tested by analysis of similarities (ANOSIM) with the Bray-Curtis similarity index. *P* value <0.05 was considered to be significant.

**2.4 Results:**

**2.4.1 Effect of CML on Weight Change and Food Intake in Healthy and Colitic Mice:**

Since both the colitis models used here are known to induce weight loss, we expressed the weight variation as the percentage of final weight on initial weight (D1 = day 1) for each animal. A result over 100 % reflects weight gain. As expected, DSS resulted in significant ($P<0.001$) weight loss as compared to control animals (92 ± 2.39 % *vs* 104.1 ± 1.25 %) and CML significantly limited the weight loss in DSS treated mice (101.3 ± 1.69 % *vs* 92 ± 2.39 %; $P<0.001$) (Table 2.1). DSS colitis was not associated with any modification of food intake. Following TNBS exposure, mice lost up to 20% of initial weight (85.09 ± 1.52% *vs* 104.1 ± 1.25 %; $p<0.001$), a loss which was associated with a significant ($P<0.001$) reduction of food consumption (2.10 ± 0.26 g *vs* 3.88 ± 0.24 g in controls) (Table 2.1). However, in this model of colitis, CML failed to limit this loss since mice lost up to 25% in comparison with the CML treated group (81.62 ± 0.82 % *vs* 107.2 ± 1.41 % in controls; $P<0.001$), while mice had a normal food intake (2.48 ± 0.41 g *vs* 3.57 ± 0.21 g in the CML group) (Table 2.1).

**2.4.2 Effect of CML on Macroscopic Lesions and Neutrophil Infiltration in Healthy and Colitic Mice:**

CML administration during three weeks did not induce any macroscopic lesions (Table 2.1). By contrast, DSS colitis was associated with significant ($P<0.001$) lesions (3.12 ± 0.29 AU *vs* 0.17 ± 0.16 AU in controls), which were not limited by ingestion of CML (3.69 ± 0.16 AU *vs* 3.12 ± 0.29 AU in the DSS group) (Table 2.1). Significant lesions ($P<0.01$) were also observed in mice submitted to TNBS colitis (6.30 ± 0.89 AU *vs* 0.17 ± 0.17 AU in controls), which were also

not limited by CML ingestion (7.75 ± 0.39 AU *vs* 6.3 ± 0.89 AU in the TNBS group) (Table 2.1). Furthermore, daily exposure to CML did not result in any neutrophil infiltration. However, MPO activity was significantly increased ($P$<0.01) after DSS (0.77 ± 0.07 U/mg *vs* 0.25 ± 0.02 U/mg de proteins in controls) and TNBS (0.64 ± 0.07 U/mg *vs* 0.25 ± 0.02 U/mg proteins) was exposure as expected (Table 2.1). In mice submitted to either DSS or TNBS, CML repeated ingestion was unable to limit the inflammatory reaction (0.70 ± 0.07 U/mg *vs* 0.77 ± 0.07 U/mg proteins in DSS group and 0.54 ± 0.06 U/mg *vs* 0.64 ± 0.07 U/mg proteins in the TNBS group) (Table 2.1).

### 2.4.3 Gut Microbiota Analyses:

A total of 2,102,977 sequences from 123 samples (17097 ± 10992 per sample) were obtained.

#### 2.4.3.1 Impact of CML on the Gut Microbiota of Healthy Mice:

At the phylum level, CML consumption did not affect gut microbiota (Figure 2.1A). Using multivariate analyses at different taxonomic levels, only slight (not statistically significant by ANOSIM) differences in gut microbiota were found between control and CML mice gut microbiota at the genus level (Figure 2.1A). However, CML consumption significantly increased the relative abundance of several Bacteroidetes families (Bacteroidaceae, Odoribacteraceae and the numerically marginal Porphyromonadaceae) (Figure 2.1B), as well as, the Desulfovibrionaceae (Proteobacteria) (Figure 2.1D) and *Dorea* (Firmicutes) (Figure 2.1C). CML consumption resulted in a significant decrease in Lachnospiraceae (Firmicutes) (Figure 2.1C) and the low abundant *Sutterella* (Proteobacteria) (Figure 2.1D).

#### 2.4.3.2 Impact of TNBS and DSS on the Gut Microbiota:

At the phylum level, similar trends were observed for TNBS and DSS treatments (Figure 2.2A). As indicated on the pie charts, DSS treatment resulted in a significant decrease of Firmicutes

(from 77 to 34%) and increase in Bacteroidetes (from 20 to 41%) and Proteobacteria (from 1 to 12%). TNBS treatment resulted in a significant decrease of Firmicutes (from 77 to 39%) and an increase in Bacteroidetes (from 20 to 42%) and Proteobacteria (from 1 to 14%). On multivariate analyses at different taxonomic levels, significant differences were observed at all taxonomic levels between control, TNBS, and DSS groups (Figure 2.2A; ANOSIM $P<0.05$ for each comparison). Consequently, the different treatments led to distinct dysbiotic microbiota compositions.

DSS treatment resulted in a sharp significant increase of Bacteroidaceae and Porphyromonodaceae, while Prevotellaceae were significantly depleted (Figure 2.2B). Lachnospiraceae, but not Ruminococcaceae, were also decreased (Figure 2.2C). DSS treatment resulted in significant ($P<0.05$) increases of a wide range of Proteobacteria members (Figure 2.2D), with a much less marked increase in Enterobacteriaceae than under TNBS (Figure 2.2D), as well as a notable increase in Actinomycetales (Figure 2.2E).

TNBS treatment also resulted in a sharp significant increase of Bacteroidaceae and Prevotellaceae (Figure 2.2B), and somewhat unexpectedly *Lactobacillus* and *Enterococcus* (Figure 2.2C). It decreased very significantly ($P<0.05$) the relative abundance of Lachnospiraceae and Ruminococcacceae (Figure 2.2C).The significant ($P<0.05$) increase in Proteobacteria induced by TNBS was solely due to a massive increase of Enterobacteriaceae reaching 13.5% (Figure 2.2D), but other Proteobacteria were not affected or even decreased significantly (Desulfovibrionaceae) (Figure 2.2D). Finally, TNBS significantly ($P<0.05$) reduced Coriobacteriacease ($P<0.05$), while had no incidence on Actinomycetales (Figure 2.2E).

#### 2.4.3.3 Impact of CML on the Gut Microbiota of DSS and TNBS-Treated Mice:

At the phylum level, no effect of CML was observed (Figure 2.3) in TNBS-treated mice, with Firmicutes slightly decreasing (39 to 36%), Bacteroidetes stable (42%) and Proteobacteria slightly increasing (14 to 18%) as indicated on pie charts. In contrast, CML consumption significantly modulated the gut microbiota towards the control/CML state, with increased Firmicutes (34 to 58%), and decreased Bacteroidetes (41 to 32%) and Proteobacteria (12 to 3%). On multivariate analyses at different taxonomic levels, no differences were observed between control and CML mice exposed to TNBS colitis (Figure 2.3). On the other hand, very significant (ANOSIM $p<0.05$) differences between control and CML mice exposed to DSS colitis was observed (Figure 2.3). For the TNBS mice, no significant difference was observed for genera representing more than 0.001% of the total microbiota (Figure 2.4B and 2.4C). Intriguingly, CML consumption appeared to result in a numerical increase of Enterobacteriaceae (Figure 2.4D) and to some extent Bacteroidaceae (Figure 2.4A).

CML consumption significantly ($P<0.05$) reverted the increase in Bacteroidaceae and Porphyromonodaceae (Figure 2.5A) and Actinomycetales (Figure 2.5C) due to DSS treatment. It also decreased the abundance of all Proteobacteria groups associated with DSS-treatment, with the exception of Desulfovibrionaceae (Figure 2.5D). These trends were associated with increases in Lachnospiraceae, Ruminococcaceae and other Clostridiales (Figure 2.5B).

## 2.5 Discussion and Conclusion:

Maillard reaction products (MRPs) have been subject of negative perception by consumers due to early reports of detrimental effects [14, 47, 48]. Acrylamide, an AGE well known as a carcinogen, for which there is substantial evidence for detrimental health impacts, has been

associated with ocular and renal diabetic complications [22-25, 49, 50]. However, novel evidence has suggested that MRPs, due to the wide range of compounds that can be formed, should not be considered as a collective of detrimental class of molecules. In fact, beneficial impacts on health, and potential antioxidant properties have been reported [51, 52]. We report herein that repeated oral ingestion of CML, a major marker of MRPs in food, does not compromise mucosal integrity of the colon since we observed neither macroscopic lesions nor colonic inflammation in healthy mice exposed to CML during 21 days. CML has been commonly used as a biomarker for AGEs in food and in circulatory blood. However, little is known about its effects on health. In high-fat diet fed rats, CML consumption led to an increase in LDL, fasting glucose and energy expenditure, and to the alteration of liver and renal function[53]. Here we did not observe any impact of CML consumption on mice general health, but it should be noted that the dose of CML we used was 30 times lower than in the previous study and is more representative of a regular daily exposure, and that the animals were fed a standard diet.

We also report that repeated consumption of moderate amounts of CML has limited impacts on the taxonomic composition of gut microbiota of normal healthy mice. The impact of MRPs on gut microbiota has been scarcely studied, mainly *in vitro* studies. In fact, only very recent studies have shown that gut microbiota can degrade selected glycated amino acids, including CML [34], and metabolites from MRP bacterial degradation could not be characterized with available Mass Spectrometry instruments. A bifidogenic impact was reported in another *in vitro* assay with an Amadori product resulting from a protein glycated with galactose, lactulose, and galacto-oligosaccharides [54]. However, this result is more probably due to the known bifidogenic impact of those “prebiotic” polysaccharides. In the present work, we observed a limited impact of CML consumption on the murine gut microbiota. It appears that CML was preferentially fermented by

*Bacteroides* species, which may be in line with previous reports of *Bacteroides* growth from advanced MRPs [55] and bread crusts rich in some melanoidins [56]. The significant increase of *Desulfovibrio* further suggests that CML stimulated fermentation rates in the mouse cecum [4, 57]. Nonetheless, it should be noted that the observed changes were modest numerically, possibly indicating that CML can be fermented by a relatively wide diversity of bacterial species.

In this study, we also compared the dysbiosis TNBS versus DSS. Gut microbiota unbalance is a known contributor of IBD [58, 59], and also suspected to be directly involved in the inflammatory process of both these chemically induced colitis models [11]. Furthermore, links between gut microbiota disruption and diet are regularly suggested in the literature. However, somewhat surprisingly, there is little knowledge regarding typical gut microbiota responses to DSS or TNBS insult, since those models are commonly used in conjunction with other parameters (diet, genetic alteration, etc.). DSS and TNBS are two of the most popular chemicals used to mimic human inflammatory bowel disease in mice. DSS treatment results in chronic intestinal inflammation by disruption of the colonic epithelial barrier, resulting in Ulcerative Colitis (UC)-like damages and immune response [41]. It has also been shown to be suitable to study epithelial repair mechanisms [60]. On the other hand, TNBS initiates acute IL-12 driven (Th1 immune response emphasis) intestinal inflammation, more similar to Crohn's Disease (CD) symptoms and etiology [61]. The TNBS model is useful to study T helper cell-dependent mucosal immune responses [62] and many important aspects of gut inflammation, including cytokine secretion patterns, mechanisms of oral tolerance, and cell adhesion. Recent literature has been reported that both models result in increased abundance of Enterobacteriaceae and *Bacteroides* [11], with *Clostridium* spp. and *Akkermansia spp.* also reported for DSS [63]. We report herein strongly contrasting alteration of gut microbiota between TNBS and DSS models. As already shown in a

previous work, we indeed observed Enterobacteriaceae and Bacteroidaceae (mainly *Bacteroides*) as the main groups involved in TNBS colitis [44]. Their increase was correlated with a sharp decrease of Lachnospiraceae and Ruminococcaceae, two of the families most commonly associated with production of anti-inflammatory short-chain fatty acids (butyrate in particular). Remarkably, *Lactobacillus* and *Enterococcus*, two genera generally considered beneficial also increased significantly in TNBS-exposed mice. *Lactobacillus spp.* in particular are known for their ability to control pathogenic Enterobacteriaceae numbers, associated with the induction/progress of the inflammatory process. Since Enterobacteriaceae were clearly not inhibited, it is likely that *Lactobacillus* absolute numbers did not increase, but were maintained relative to other taxa. DSS treatment only resulted in a modest increase in Enterobacteriaceae, but also in a general increase of a wide range of Proteobacteria, suggesting an increased role for nonpathogenic species in inflammatory response. On the other hand, DSS also resulted in less marked depletion of Firmicutes, with maintenance of Ruminococcaceae levels. In slurries derived from Ulcerative Colitis patients, glycated bovine serum albumin was found to increase the numbers of *Clostridia*, sulfate-reducing bacteria and *Bacteroides* with the decrease of *Bifidobacteria*[64]. Overall, it appears that the bacterial dysbiosis incurred by DSS in comparison to the TNBS is due to the different course of the inflammatory process.

CML consumption had virtually no impact on the gut microbiota dysbiosis and on the inflammatory reaction of TNBS-treated mice. In contrast, CML limited the severity of weight loss and the gut microbiota dysbiosis in DSS-treated mice, but not the inflammatory response. Overall, these data suggest that CML has the potential to improve the DSS dysbiosis, by incurring more significant changes than in the healthy microbiota. Remarkably, the changes in Bacteroidetes and Firmicutes brought by CML in DSS animals were opposite to those observed in healthy mice.

Firmicutes, specifically Lachnospiraceae, Ruminococcaceae (*Oscillospira* and *Ruminococcus* being the most abundant genera) and Clostridiales, were strongly stimulated by CML consumption only in DSS-treated mice, while initial levels were comparable in both models. While *Oscillospira* is still a poorly understood member of the mammalian gut microbiota, there is evidence that they can consume host-derived glycoproteins [65]. Thus, they may be primary degraders of dietary CML as well as other MRPs. The beneficial effect of CML in this case probably results from the control of the Proteobacteria bloom, which may be direct [35] or indirect through competitive exclusion. The observation of opposite dynamics for Bacteroidaceae (*Bacteroides*) after CML consumption demonstrates that different species are stimulated by DSS and TNBS, and that the species involved in DSS colitis may not be able to utilize CML as well as those associated with TNBS colitis.

Overall, we show here that consumption of CML, a surrogate of Advanced Glycation End-products, has little impact on healthy gut microbiota, but alleviates gut microbiota dysbiosis in DSS-treated mice only. These data confirm that determining the health effects of MRPs is more complex than it would seem. They cannot be analyzed as a whole but rather studied one by one under clearly specified conditions. We may also suggest that the response of animals could be different if CML was given in already colitic animals. It is possible that selected MRPs may be used for preventive purposes, with anti-inflammatory potential mediated by gut microbiota. However, a better understanding of MRPs fate in the gut microbiota environment will be needed before suggesting any health claims. Their effects on altered intestinal mucosa needs further investigation to make food heating recommendations especially for patients suffering from chronic inflammation.

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**Conflicts of interest:**

The authors declare that they have no conflict of interest

**Ethical Approval:**

All applicable national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institutions or practice at which the studies were conducted.

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**Table 2.1:** Effect of $N^{\varepsilon}$Carboxymethyllysine treatment on weight change, food intake, macroscopic lesions, MDS and myeloperoxidase activity, MPO (U/mg protein) observed following either DSS or TNBS experimental colitis:

| | **Control** | **CML** | **DSS** | **DSS+CML** | **TNBS** | **TNBS+CML** |
|---|---|---|---|---|---|---|
| Weight change (% Day 1) | 104.1± 1.25 | 107.2 ±1.41 | 92 ± 2.39 [a] | 101.3 ± 1.69 [c] | 85.09 ± 1.52 [a] | 81.62 ± 0.82 [b] |
| Food intake (g/d/mouse) | 3.88 ± 0.24 | 3.57 ± 0.21 | 3.14 ± 0.26 | 3.77 ± 0.23 | 2.1 ± 0.26 [a] | 2.48 ± 0.41 |
| Macroscopic damage scores (AU) | 0.17 ± 0.16 | 0.71 ± 0.36 | 3.12 ± 0.29[a] | 3.69 ± 0.16[b] | 6.3 ± 0.89[a] | 7.75 ± 0.39[b] |
| MPO activity (U/mg protein) | 0.25 ± 0.02 | 0.23 ± 0.05 | 0.77 ± 0.07[a] | 0.7 ± 0.07 [b] | 0.64 ± 0.08 [a] | 0.53 ± 0.06 [b] |

Weight change corresponds to the ratio of final weight on Day 1 weight (in %). Food intake illustrates the amount in grams (g) eaten per animal and per day. Macroscopic lesions were illustrated as macroscopic damage scores (MDS) and expressed in arbitrary units (AU). MPO activity is expressed in U/mg of total protein content in the sample. Data are expressed as mean ± SEM. A *P* value <0.05 was considered to be significant.

[a] significantly different ($P<0.05$) from control.

[b] significantly different ($P<0.05$) from the CML group.

[c] significantly different ($P<0.05$) from the DSS group. DSS and TNBS colitis induced significant ($P<0.05$) weight loss, macroscopic damage and increase of MPO activity. This was partially limited in DSS colitic mice treated with CML but not in TNBS colitic mice.

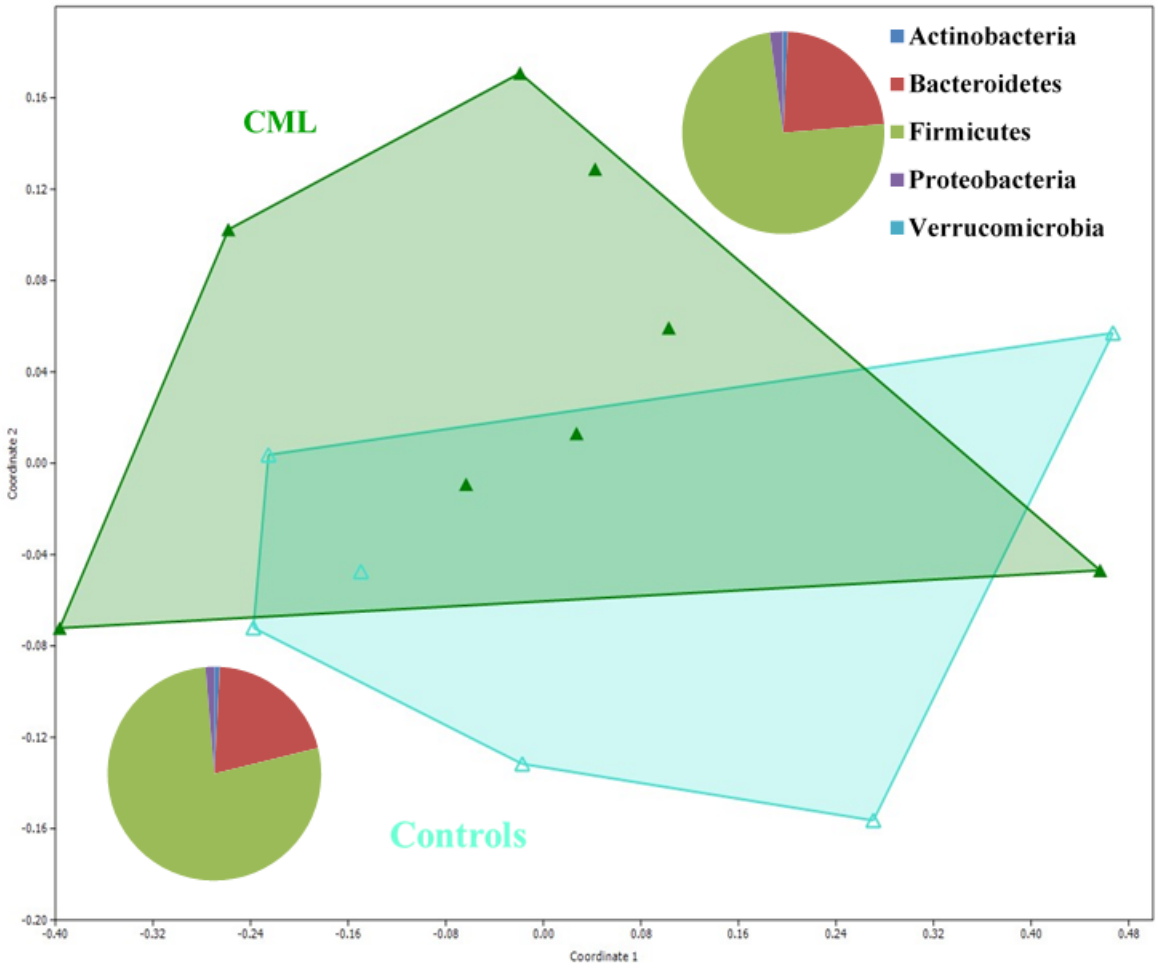
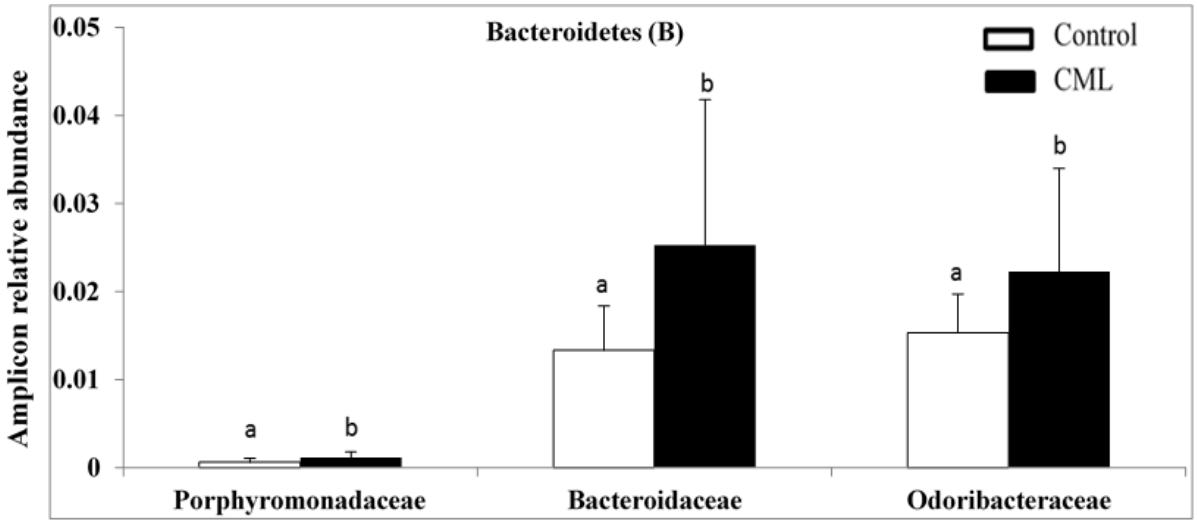
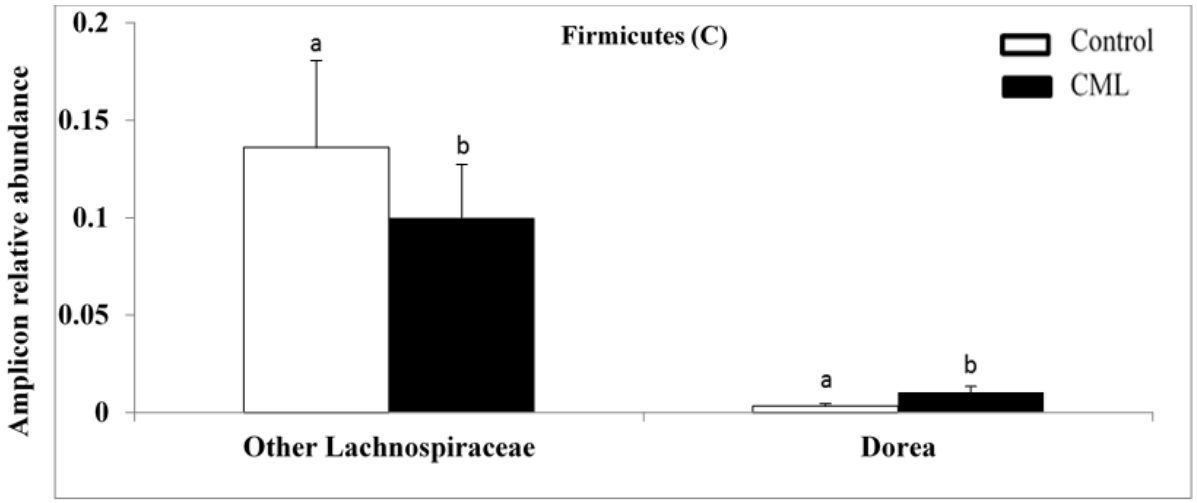



**Figure 2.1A: $N^{\varepsilon}$-Carboxymethyllysine (CML) consumption results in modest and inconsistent changes in the healthy mice gut microbiota**. Microbiota data are shown on a non-metric multidimensional scaling (Bray-curtis similarity index; ANOSIM: $P>0.05$) and as pie charts.


Bacteroidetes (B)
Control
CML
Amplicon relative abundance
0.05
0.04
0.03
0.02
0.01
0
a
b
a
b
a
b
Porphyromonadaceae
Bacteroidaceae
Odoribacteraceae


Firmicutes (C)
Control
CML
Amplicon relative abundance
0.2
0.15
0.1
0.05
0
a
b
a
b
Other Lachnospiraceae
Dorea

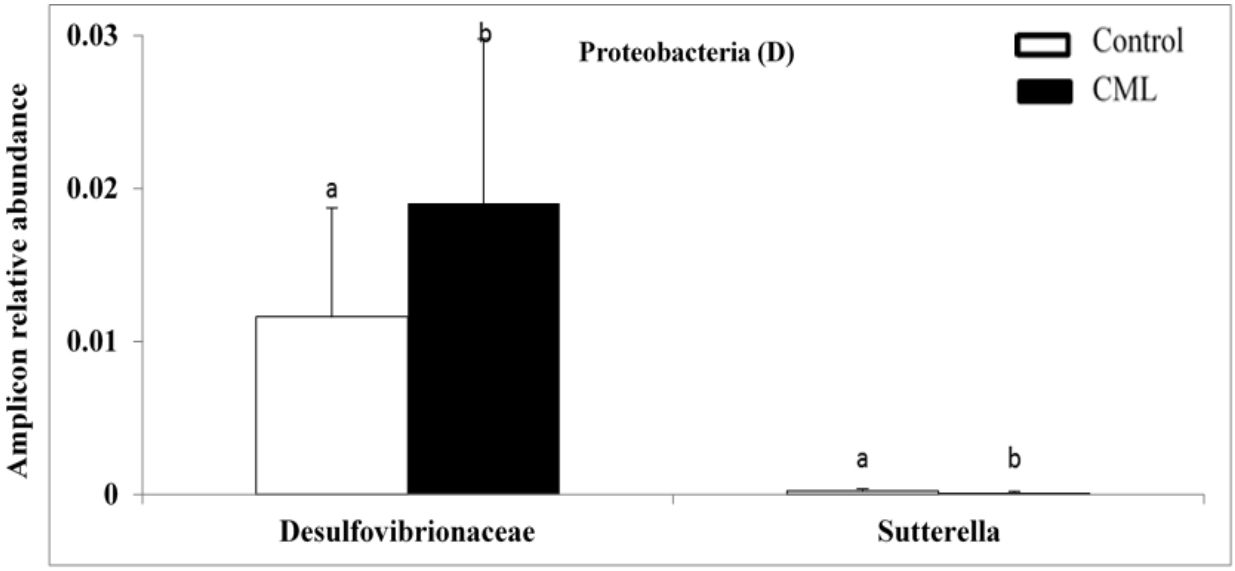



**Figure 2.1B-D: $N^{\varepsilon}$-Carboxymethyllysine (CML) consumption results in modest and inconsistent changes in the healthy mice gut microbiota:** B-D Relative abundance of mice gut microbiota composition at the phylum level: (B) Bacteroidetes, (C) Firmicutes, and (D) Proteobacteria. Only taxa representing more than 0.001% of the gut microbiota were considered. Results are expressed as mean ± SEM (Kruskal Wallis). Statistical differences ($P$<0.05) are represented by different letters. Repeated CML exposure does not change the overall gut microbiota composition at the phylum level. However, within the phyla studied there was a redistribution of the populations.

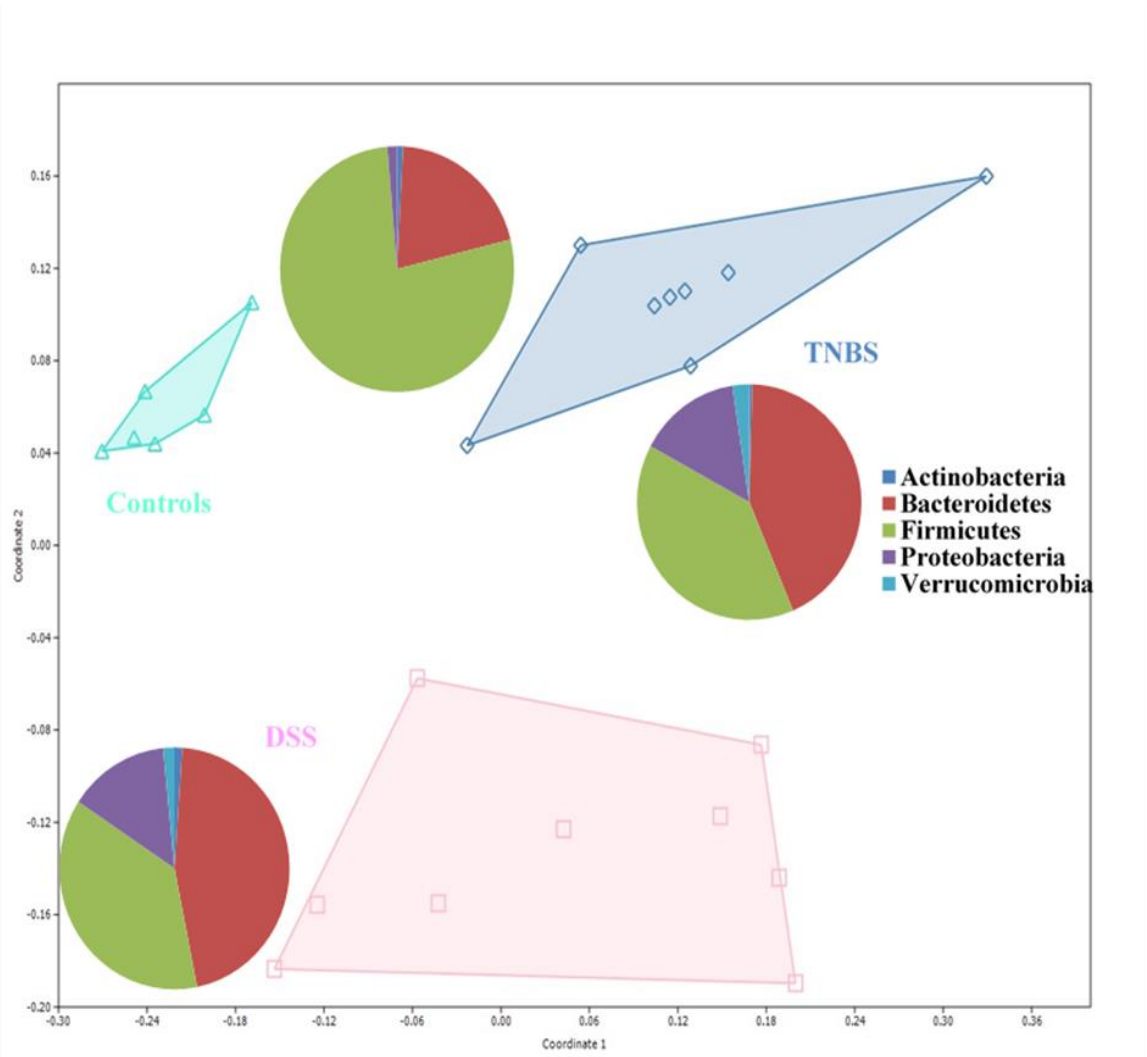
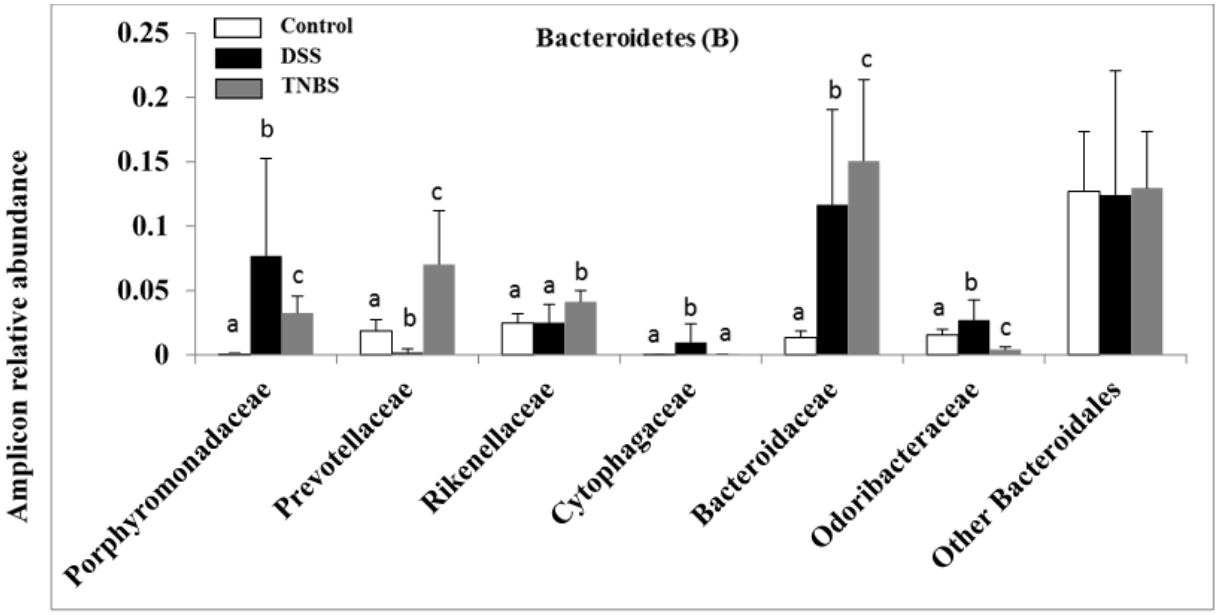
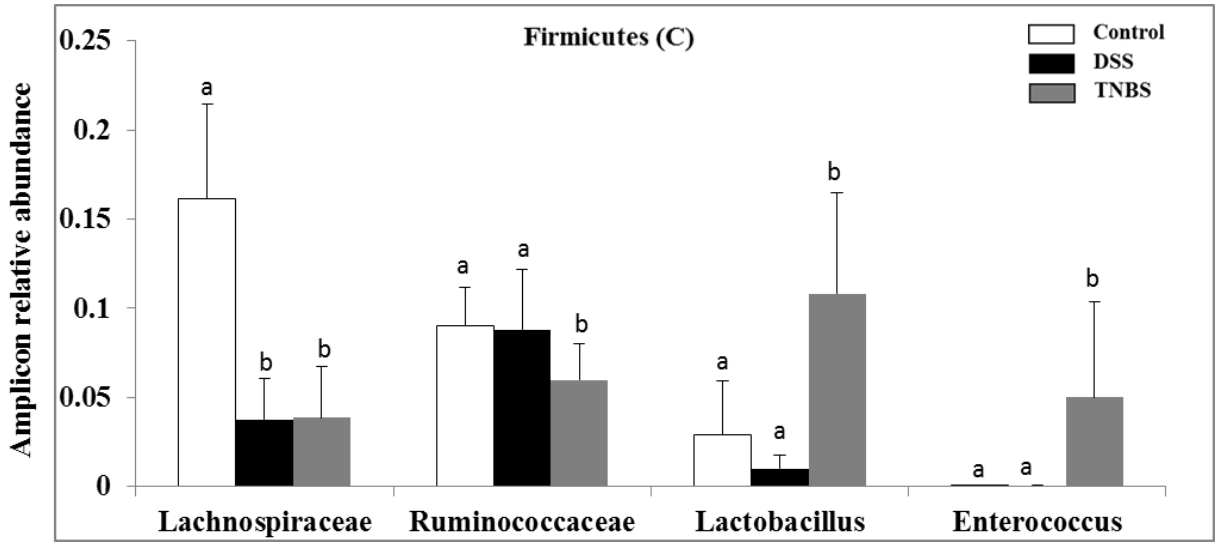
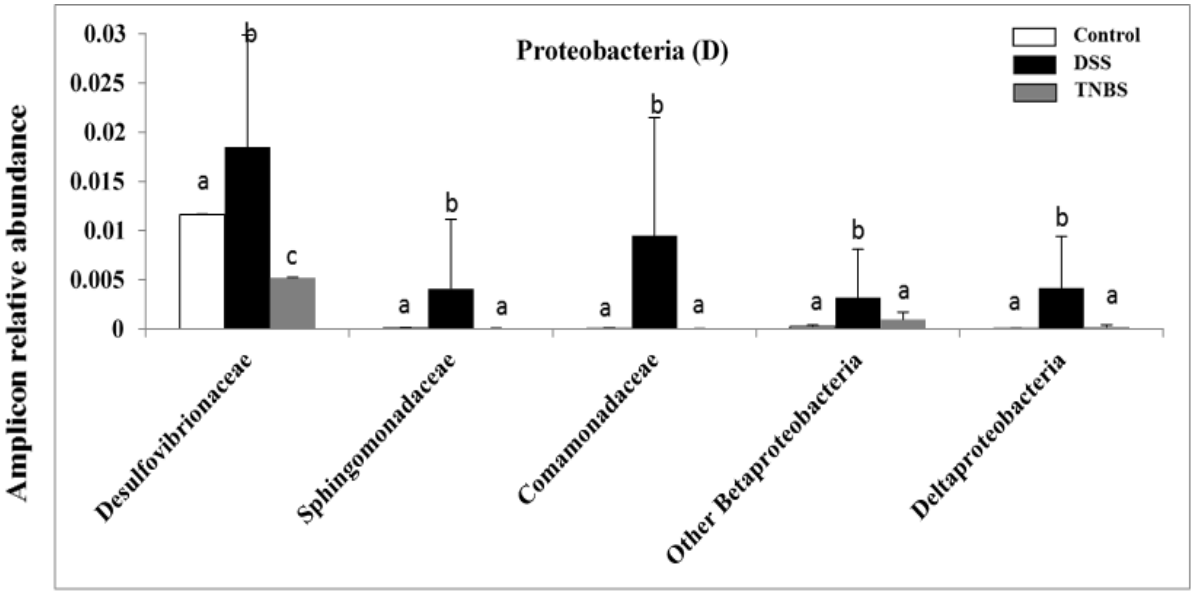
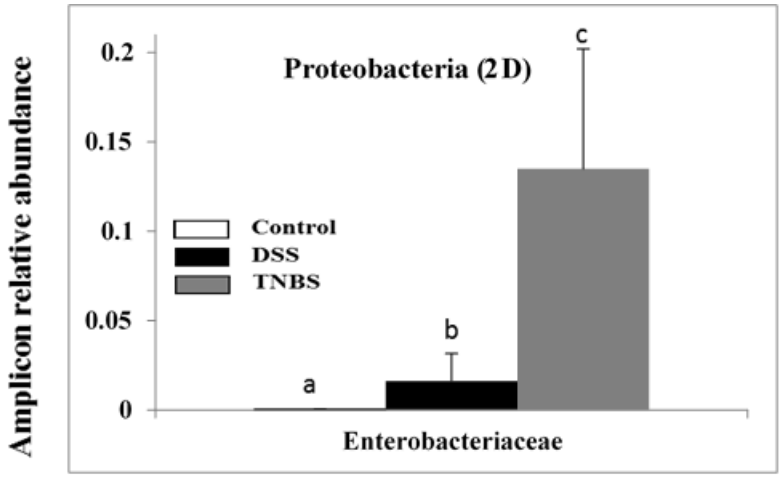



**Figure 2.2A: DSS and TNBS experimental colitis results in dramatic and distinct alterations of the healthy mice gut microbiota** Microbiota data are shown on a non-metric multidimensional scaling (Bray-curtis similarity index; ANOSIM: $P<0.05$) and as pie charts.


Bacteroidetes (B)
Control
DSS
TNBS
Amplicon relative abundance
0.25
0.2
0.15
0.1
0.05
0
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Cytophagaceae
Bacteroidaceae
Odoribacteraceae
Other Bacteroidales


Firmicutes (C)
Control
DSS
TNBS
Amplicon relative abundance
0.25
0.2
0.15
0.1
0.05
0
Lachnospiraceae
Ruminococcaceae
Lactobacillus
Enterococcus


Proteobacteria (D)
Amplicon relative abundance
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
DSS
TNBS
a
b
b
c
a
b
a
a
b
a
a
b
a
b
a
a
b
a
Desulfovibrionaceae
Sphingomonadaceae
Comamonadaceae
Other Betaproteobacteria
Deltaproteobacteria


Proteobacteria (2D)
Amplicon relative abundance
0.2
0.15
0.1
0.05
0
Control
DSS
TNBS
a
b
c
Enterobacteriaceae

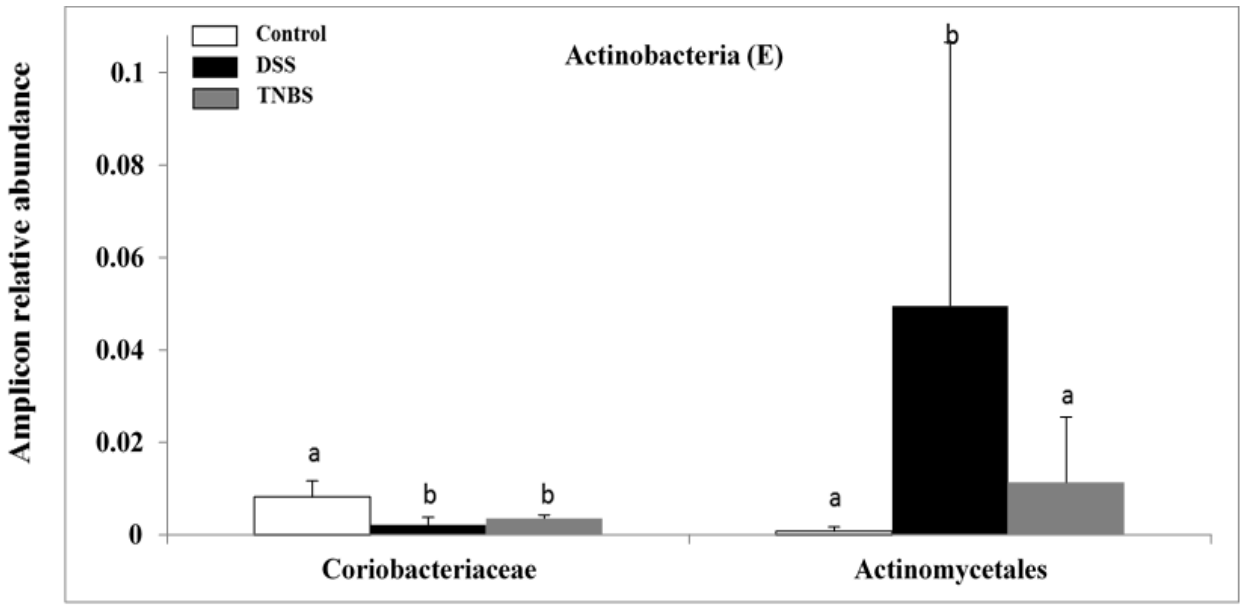



**Figure 2.2B-E: DSS and TNBS experimental colitis results in dramatic and distinct alterations of the healthy mice gut microbiota** Relative abundance of mice gut microbiota composition at the phylum level. Significant and specific dysbiosis induced by experimental colitis impacts several specific bacterial groups (Kruskal-Wallis test: $P<0.05$). Only taxa representing more than 0.001% of the gut microbiota were considered: Bacteroidetes (B); Firmicutes (C); Proteobacteria (D) and Actinobacteria (E). Enterobacteriaceae are presented separately in insert on 2D, as their relative abundance increases dramatically in TNBS mice. Results are expressed as mean ± SEM (Kruskal Wallis). Statistical differences ($P<0.05$) are represented by different letters. Significant and specific dysbiosis induced by experimental colitis impacts several specific bacterial groups (Kruskal-Wallis test: $P<0.05$) leading to an increase of Proteobacteria and Bacteroidetes and a decrease of Firmicutes.

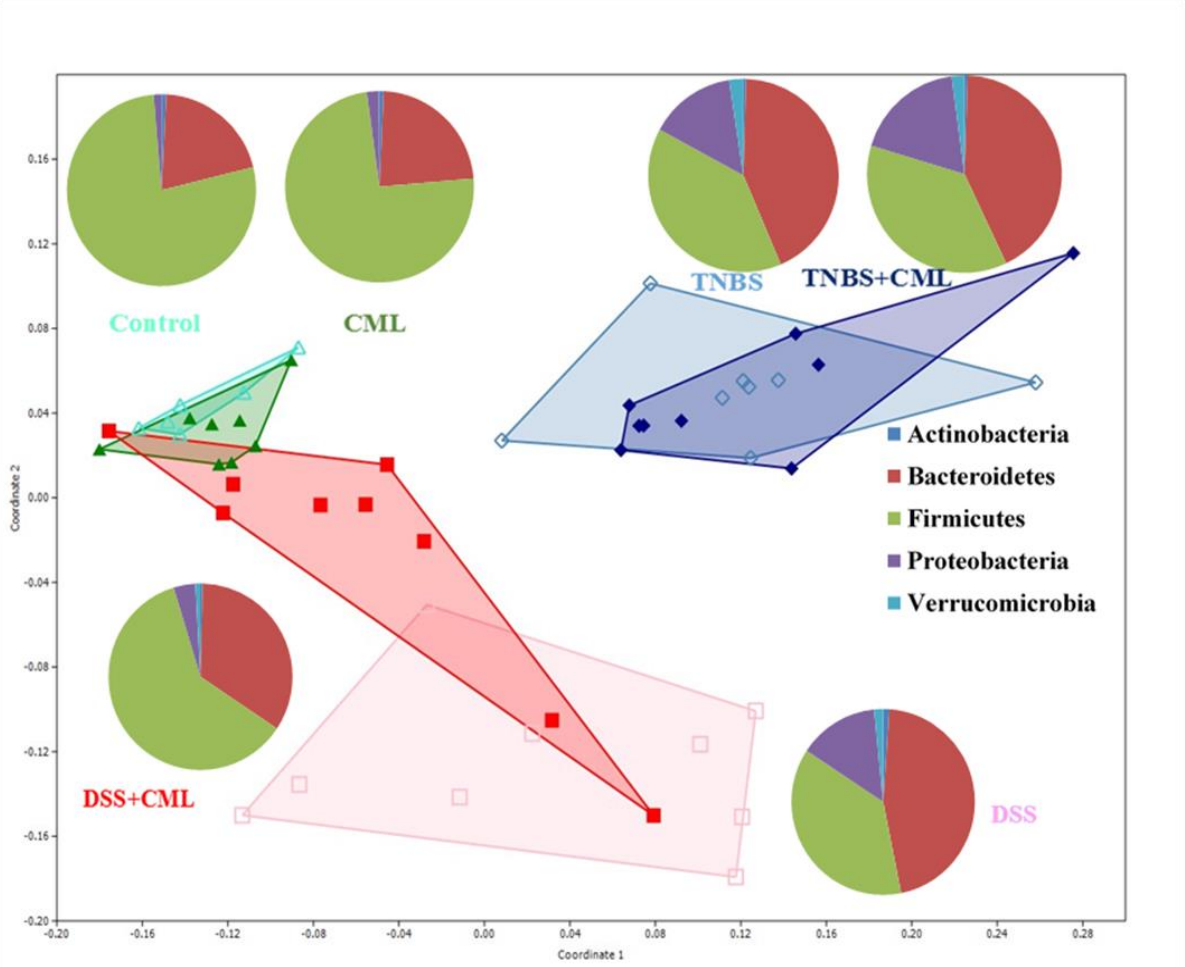
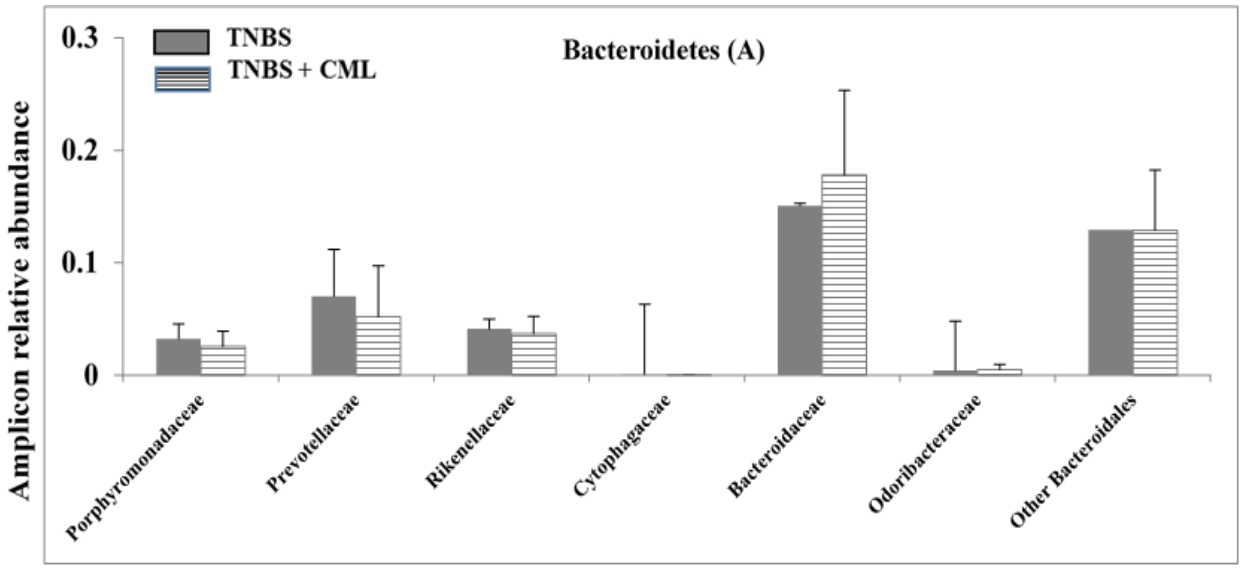
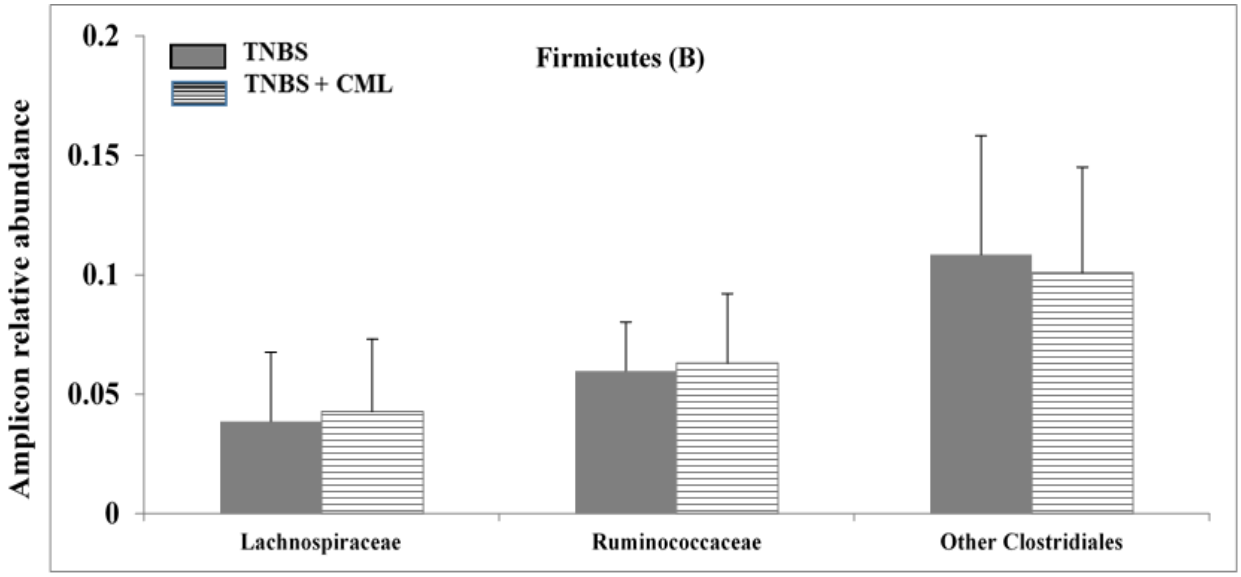



**Figure 2.3: CML differentially modulates the limitation of DSS and TNBS alterations of the mice gut microbiota.** Microbiota data are shown on a non-metric multidimensional scaling (Bray-curtis similarity index; ANOSIM: $P>0.05$) and as pie charts. CML limites the alteration under DSS colitis but not on TNBS colitis.


Bacteroidetes (A)
TNBS
TNBS + CML
Amplicon relative abundance
0.3
0.2
0.1
0
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Cytophagaceae
Bacteroidaceae
Odoribacteraceae
Other Bacteroidales


Firmicutes (B)
TNBS
TNBS + CML
Amplicon relative abundance
0.2
0.15
0.1
0.05
0
Lachnospiraceae
Ruminococcaceae
Other Clostridiales

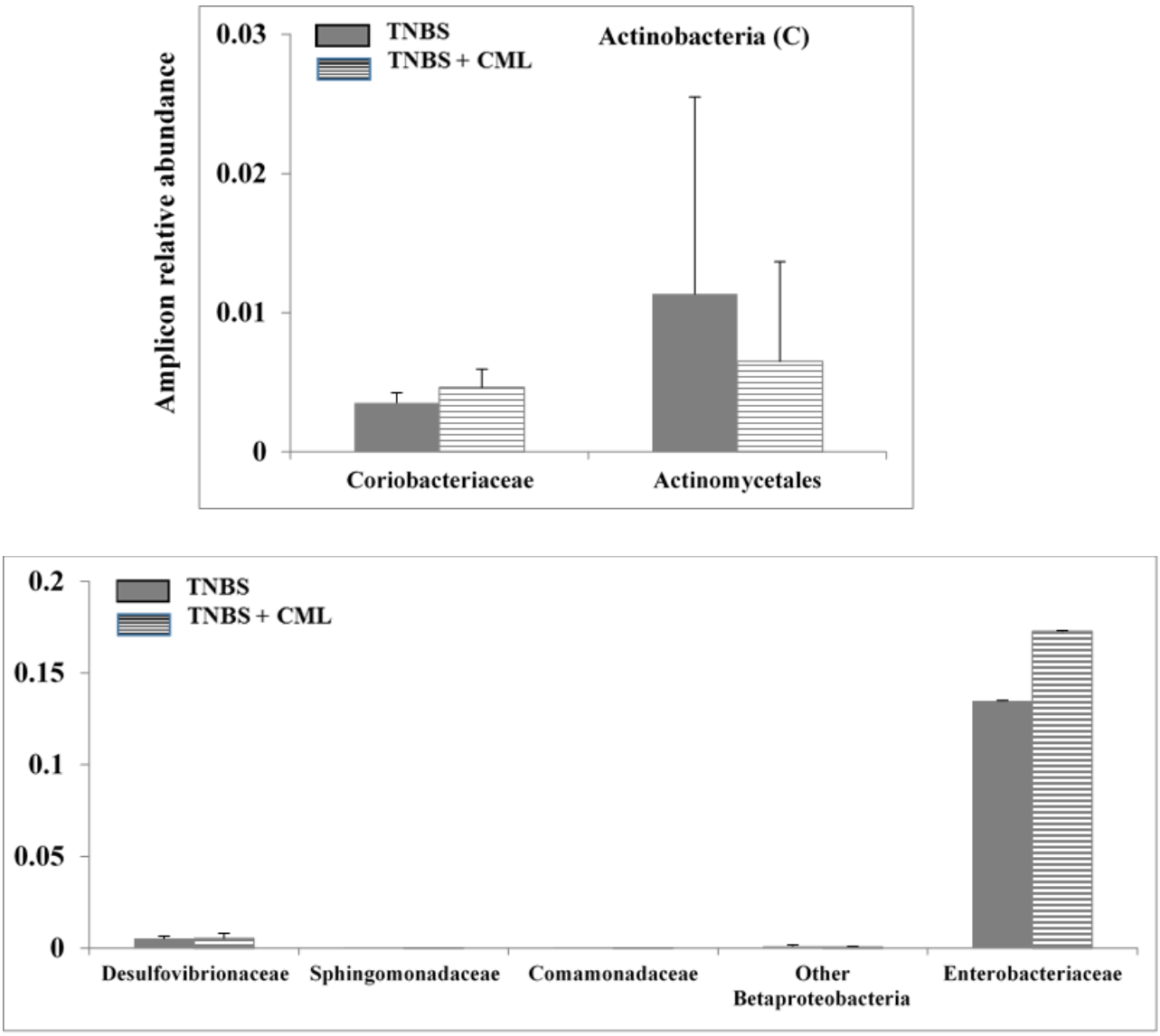
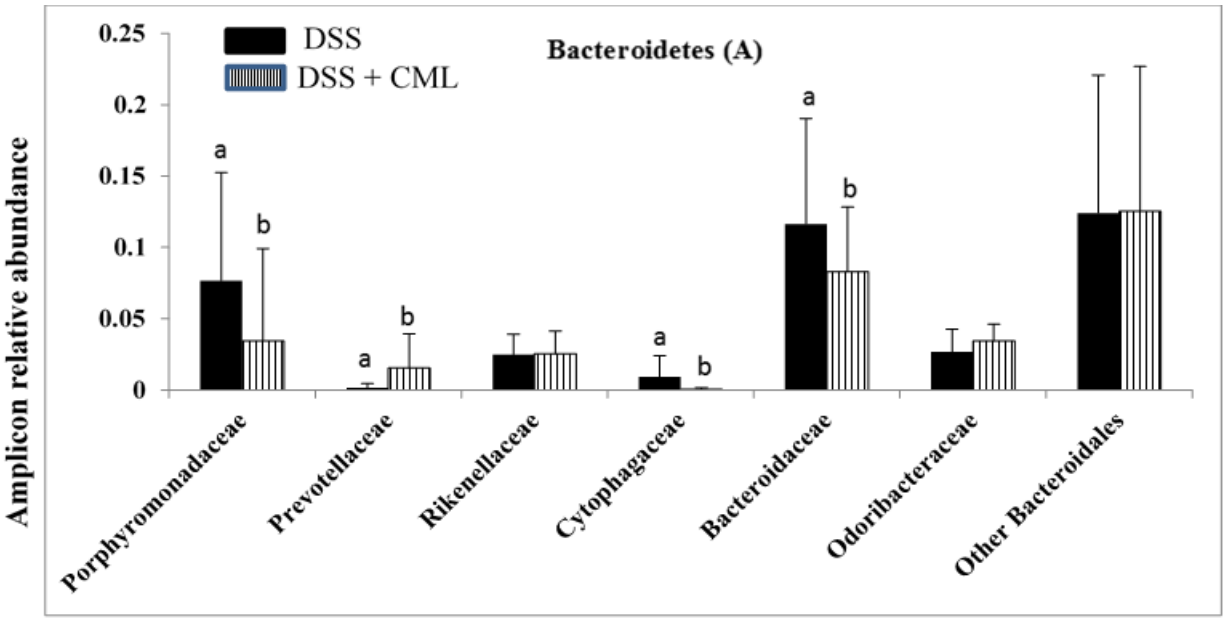
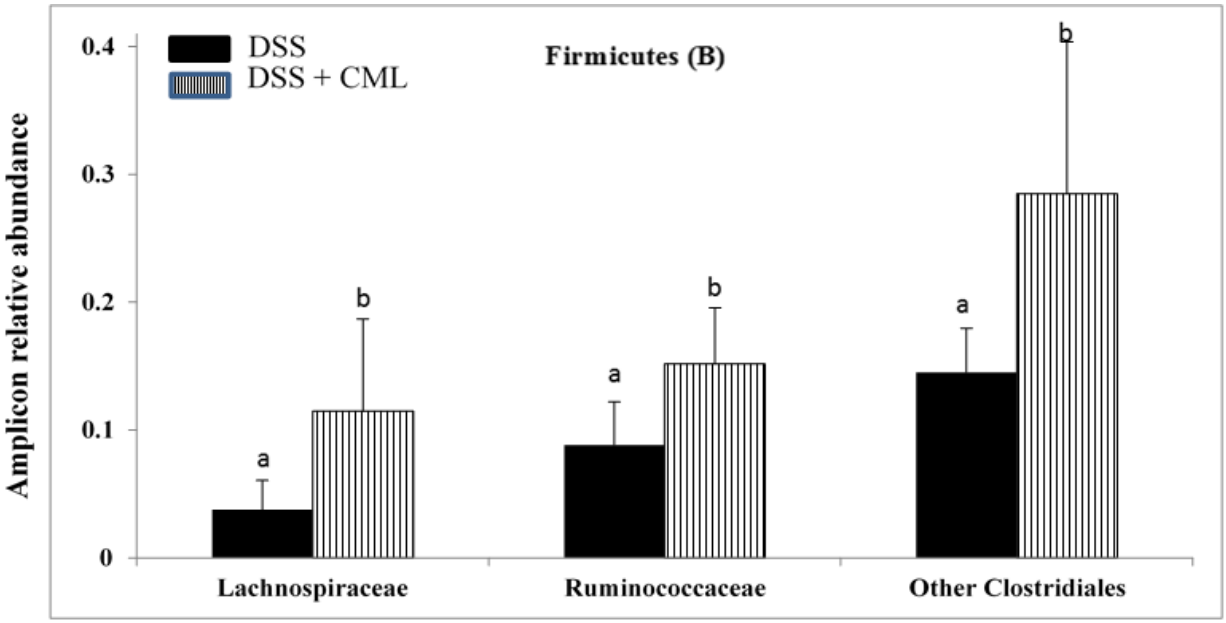



**Figure 2.4A-D: CML has a limited impact on TNBS-induced gut microbiota dysbioses.** Relative abundance of mice gut microbiota composition at the phylum level: Bacteroidetes (A); Firmicutes (B); Actinobacteria (C), Proteobacteria (D). Only taxa representing more than 0.001% of the gut microbiota were considered. Results are expressed as mean ± SEM (Kruskal Wallis). CML has no significant impact on TNBS-induced gut microbiota dysbiosis while it increases numerically Bacteroidaceae and Enterobacteriaceae).


Bacteroidetes (A)
DSS
DSS + CML
Amplicon relative abundance
0.25
0.2
0.15
0.1
0.05
0
a
b
a
b
a
b
a
b
a
b
a
b
Porphyromonadaceae
Prevotellaceae
Rikenellaceae
Cytophagaceae
Bacteroidaceae
Odoribacteraceae
Other Bacteroidales


Firmicutes (B)
DSS
DSS + CML
Amplicon relative abundance
0.4
0.3
0.2
0.1
0
a
b
a
b
a
b
Lachnospiraceae
Ruminococcaceae
Other Clostridiales

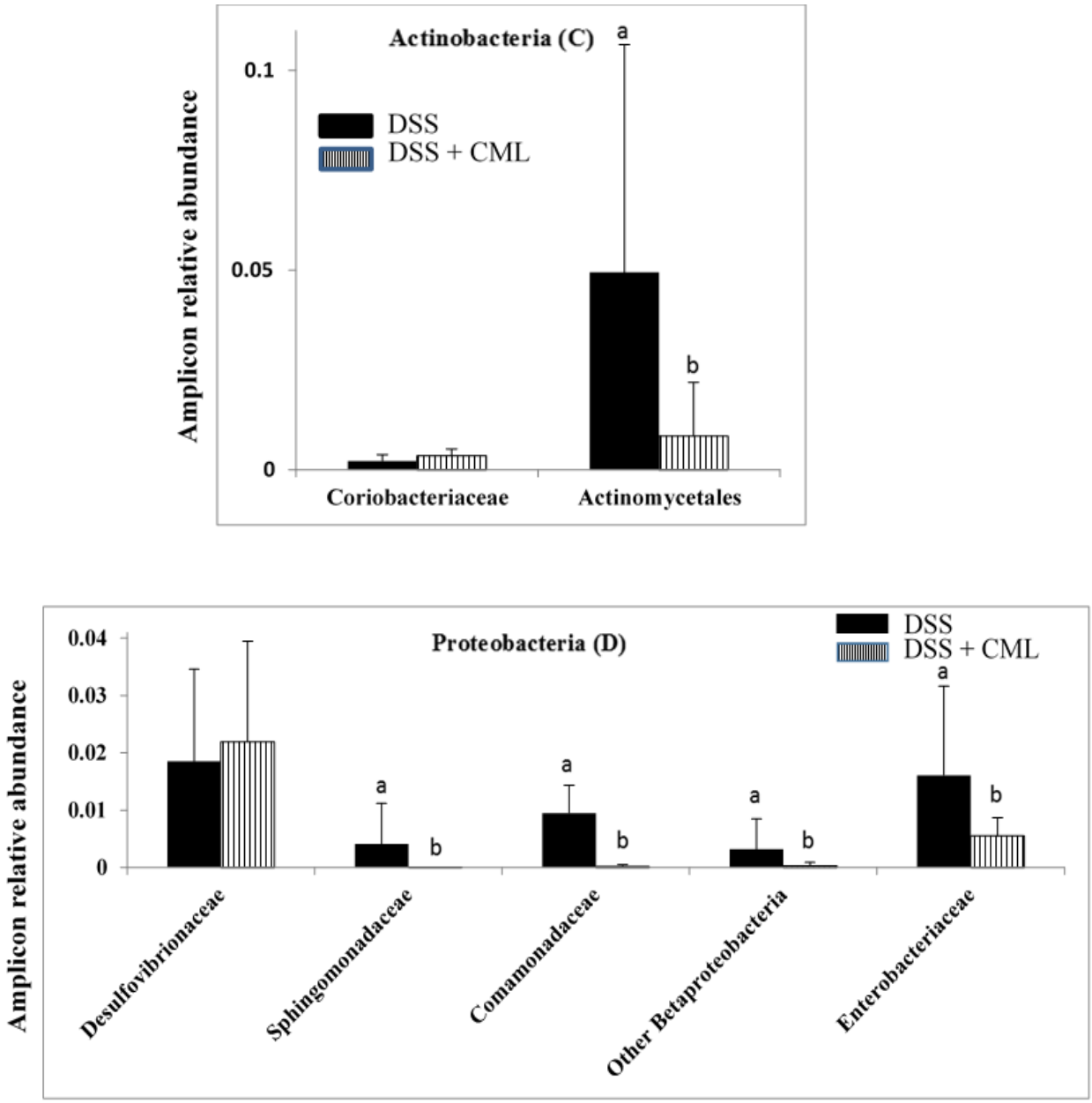



**Figure 2.5A-D: CML consumption significantly modulates DSS-treated mice gut microbiota.** . Relative abundance of mice gut microbiota composition at the phylum level: Bacteroidetes (A); Firmicutes (B); Actinobacteria (C) and Proteobacteria (D): Only taxa representing more than 0.001% of the gut microbiota were considered. Results are expressed as mean ± SEM (Kruskal Wallis). Statistical differences ($P$<0.05) are represented by different letters. CML limits DSS-induced gut microbiota dysbiosis.

CHAPTER THREE

RESEARCH ARTICLE

# Impact of melanoidins from bread crust model on the gut microbiota of healthy and experimental low-grade colitis model rats

ALJahdali Nesreen,[1*] Helou Cynthia,[,2,3] Anton Pauline M,[2] Tessier Frederic J,[2,4] Carbonero Franck,[1,5] Gadonna-Widehem Pascale[2]

[1]Cell and Molecular Biology Program; University of Arkansas, AR, USA

[2]Unité Transformation & Agro-Ressources, Institut Polytechnique Unilasalle, Beauvais, France

[3] Université Saint Joseph de Beyrouth, Faculté de Pharmacie, Département de Nutrition, Beirut, Lebanon

[4]University Lille, Inserm, CHU Lille, U995-LIRIC-Lille Inflammation Research International Center, F-59000 Lille, France

[5]Department of Food Science; University of Arkansas, AR, USA

Corresponding author: Franck Carbonero

**3.1 Abstract:**

Bread melanoidins are produced during the last stage of the Maillard reaction (MR). The high amounts of melanoidins are found in bread crust, compared to bread crumbs. Melanoidins are known to have a high molecular weight (HMW), which are indigestible and reach the large intestine. Several studies showed that melanoidins are fermented by gut microbiota. The aim of this study was to determine the effect of bread melanoidins on the composition of rat's gut microbiota and potential positive modulation of the microbiota profile associated with experimental colities. Forty eight rats received either pellet (chow), bread crust model (BCM), or melanoidin-free control (MFC) for 29 days. In the last week, each group was split into sub-groups, receiving Trinitrobenzene sulfonic acid (TNBS) to induce colitis. The consumption of BCM and MFC resulted in a significant decrease of *Bacteroides* and Enterobacteriaceae, compared to the control. However, the BCM limited to increase Enterobacteriaceae TNBS model. Significant increases were observed in *Faecalibacterium* in BCM model. In contrast, the relative abundances of *Biofidobacteria* and *Lactobacillus* were decreased in rats that consumed BCM, compared to a group that consumed MFC.

**Keywords**: Maillard reaction products, Bread Melanoidins, Gut microbiota

## 3.2 Introduction

Melanoidins are polymeric, high molecular weight, brown compounds produced by the Maillard reaction during thermal processing of foods containing amino acids and reduced sugars (HODGE, 1953). Melanoidins are particularly prevalent in the Western diet, with high amounts reported in bakery products, roasted coffee (Fogliano & Morales, 2011), roasted barley (Milic, Grujicinjac, Piletic, Lajsic, & Kolarov, 1975), and many other foods subjected to intense heating (Hofmann, Bors, & Stettmaier, 1999). The chemical structure of melanoidins depends on the types of sugars, proteins, and physical parameters, such as temperature, heating time, pH, water activity, and other factors (Martins & van Boekel, 2003). Coffee and bread melanoidins have been given more attention in research due to their nutrition and health properties (Fogliano & Morales, 2011; Hofmann et al., 1999), and several studies reported that food melanoidins possess antioxidant, antihypertensive, antimicrobial, and prebiotic properties (ALJahdali & Carbonero, 2017; Wang, Qian, & Yao, 2011).

Bread melanoidins are only found in the crust, and the amount of melanoidins measured in the crust of sourdough loaves, sliced bread, and baguette were 30, 18, and 14 / 100 g, respectively (Fogliano & Morales, 2011). The intake of bread melanoidins ranged between 1.8-15.0 g per day (Fogliano & Morales, 2011). The principal constituents of bread melanoidins are starch and proteinaceous material (Hofmann et al., 1999). However, because bread melanoidins are insoluble high molecular weight molecules, they are poorly absorbed through digestive processes and reach the large intestine mostly intact (Helou et al., 2017; Helou et al., 2015; Tagliazucchi & Bellesia, 2015). It is now well established that undigested dietary elements are subject to intense fermentation by resident microbes, and there has been extensive research on the impact of diet on the human and animal gut microbiota (O'Keefe et al., 2015; Su et al., 2015). However, the majority

of studies on the diet, and the gut microbiota have focused on the basic nutrients: carbohydrates and fibers (Martinez, Kim, Duffy, Schlegel, & Walter, 2010; Zhang et al., 2016), proteins (Louis, Hold, & Flint, 2014) and fat (Devkota et al., 2012), while Maillard reaction products in general and melanoidins in particular have been sparsely studied (ALJahdali & Carbonero, 2017). Previous studies investigated the impact of bread melanoidins on the composition of gut microbiota *in vitro*. In one *in vitro* study, bread crust melanoidins were found to increase the abundance of *Bifidobacterium* species (Borrelli & Fogliano, 2005), while a recent study *in vivo* showed that bread melanoidins had no bifidogenic effect (Helou et al., 2017). Also, an *in vitro* study showed that bread melanoidins were able to inhibit the development of Enterobacteriaceae in stool cultures (Helou et al., 2015). The *in vitro* results suggest that bread crust melanoidins may act as prebiotics; however, *in vivo* studies are crucial to strengthen conclusions on prebiotic properties. The aim of this study was to determine the impact of model bread crusts containing melanoidins or not on the composition of the gut microbiota of healthy and chemically-induced colitic rats.

## 3.3 Material and Methods

### 3.3.1 Bread Preparation

Fiber-free models of bread crust were prepared as described previously (Helou et al., 2017). Briefly, for the bread crust model (BCM) starch, gluten, D-glucose and water (52.25%, 10.5%, 2.75%, and 34.5% of the dough weight, respectively) were homogenized and kneaded with a Kenwood Cooking Chef KM089 premium (De Longhi Kenwood, Clichy, France) and baked at 220 °C for 16 min in an Air-o-Speed ventilated oven (Electrolux, France). For the melanoidin-free control (MFC), the same preparation was followed, except that gluten was heated separately to prevent melanoidin formation.

### 3.3.2 Animals and Study design

Experiments were conducted at the Animal House Unit of LaSalle Beauvais under approval from the local animal protocol review committee (C60-200-01) and the French Ministry of Higher Education, Research and Innovation (CEEA n°116 and MENESR n°1688.V02) (Helou et al., 2017). Forty eight male Sprague-Dawley rats 7 weeks old (75-99 g) purchased from Harlan Laboratories (Ganat, France) were housed in stainless steel cages under controlled temperature (21 ± 1°C) and 12H light-dark cycle with unlimited access to food and water. During the first week, all rats were fed normal A04 pellets (Safe, Epinay sur Orge, France) and tap water ad libitum. After this adaptation week, the rats were divided into three groups of 16: one received A04 pellets, the others received pellets of A04 supplemented with, BCM or MFC pellets for 29 days ad libitum. BCM and MFC pellets were obtained from a special line by mixing of A04 powder with 13% (w/w) of BCM or MFC followed by compaction (Safe, Epinay sur Orge, France). During the last week, the three groups were divided into two sub-groups (6 groups, n=8 in each group): control and Trinitrobenzene sulfonic acid (TNBS) colitis. The TNBS solution (Sigma-Aldrich) was diluted in 50% ethanol (40 mg/kg – 50 µL IR) before being injected intrarectally (IR) with a lubricated silicone tubing (4cm from the anus) under general anesthesia (ketamine/xylazine 100 mg/kg – 50% v/v – 1mL/kg i.p.). Animals were then kept head down on a heating pad until full recovery from the anesthesia.

At the end of the experiment, animals were sacrificed by cervical dislocation under general anaesthesia (ketamine/xylazine – 100 mg/kg i.p. – 50% v/v). A midline laparotomy was then completed, macroscopic lesions were assessed and pieces of the colon were immediately removed and snap frozen until further analysis. To perform the analysis of the incidence of each treatment on the microbiota profile, caeca were ligated at both the ileo-caecal and the caeco-colonic junctions

before their removal and their contents were immediately collected under anaerobic conditions and snap frozen until further determination.

### 3.3.3 DNA Extraction and PCR Amplifications (Polymerase Chain Reaction)

Genomic DNA was extracted from ceca samples. Briefly, 100 mg cecal content and 1 mL of ASL buffer (Qiagen, Courtaboeuf, France) were pooled in 2 mL Lysing Matrix E tubes (MP Biomedicals, Santa Ana, USA). After mixing for 1 min with a vortex, the tubes were heated at 95°C for 5 min in a Thermomixer (Eppendorf, Montesson, France) and were then cooled in ice for 5 min. The tubes were transferred into the FastPrep® (MP Biomedicals, Santa Ana, USA) bead-beater for 2 runs of 1 min each at 6 m/s. After centrifugation (16000g at 4℃ for 1 min), the total DNA was extracted from the obtained lysates using a commercial kit (QIAamp DNA stool Mini Kit) following the manufacturer's protocol.

Dual-index PCR targeting the V4 region of the bacterial 16S rRNA gene was performed following previously described protocols (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Briefly, PCR reactions were conducted in 27 µL reaction containing: 2 µL of DNA template; 2 µL of index primers; and 23 µL of AccuPrime™ Taq DNA Polymerase (Invitrogen, USA) following the manufacturer's protocol. Amplifications were performed by initial denaturation at 95°C for 3 min, followed by 25 cycles of denaturation at 98°C for 30 sec, primer annealing at 55°C for 30 sec, and extension at 72°C for 1 min. PCR amplicons were examined on agarose gel electrophoresis by using 6 µL of ethidium bromide fluorescent dye to confirm the success of the PCR.

### 3.3.4 Library Preparation

Normalization of the PCR products was performed with a SequalPrep kit (Invitrogen) following the manufacturer's instructions. Normalized amplicons were pooled and checked on

agarose gels. Gel extraction of the correct amplicon was accomplished by running 100 µL of DNA on an agarose gel electrophoresis, and then extracting DNA from excised bands with the Min Elute Gel Extraction kit (Qiagen, Hilden, Germany).

Library quality checks using by means of real-time q-PCR and TapeStation (Agilent®, Santa Clara, USA) were performed to know the concentration and the size of DNA libraries, respectively. Q-PCR was performed with the KAPA Library Quantification Kit (KAPA Biosystems) following the manufacturer's recommendations. The dilutions were prepared from the gel extraction by taking 5 µL of DNA and adding it to 45 µL of H2O, and dilution was created starting from: 1:10, 1:100, 1:1000, 1:2000, and 1:4000. Then, the q-PCR plate was prepared by running 4 µL triplicate of standards with 6 µL of Primer premix and 4 µL triplicate of library dilutions with 6 µL of Primer premix. The Q-PCR plate was placed in the thermocycler by following initial activation at 95 °C for 5 min and 35 cycles. Amplicon size was determined again after gel extraction on the TapeStation, which was 421pb.

3.3.5 Sequencing, Bioinformatics and Statistical Analyses

Amplicon pools were diluted to 0.70 nM with 0.2 N fresh NaOH following recommended Illumina protocols. Denatured libraries were diluted to 6 pm as a final concentration, with addition of 15 pm of Phix control V3. The diluted denatured libraries were loaded on an Illumina MiSeq sequencing cartridge V2-500 cycles, with index primer, Read 1, and Read 2 sequencing primers (Kozich et al., 2013). The runs were monitored with Sequence Analysis Viewer with particular emphasis on appropriate cluster density (900-1000k/mm2) and quality scores (final >Q30 score of >80%).

All sequences were downloaded from the Illumina Basespace server in Fastq files format. All reads were analyzed using the MOTHUR software package 1.39.5 following the Illumina SOP

script (Schloss et al., 2009). All reads were carried out using the SILVA database as a reference for assignation of operational taxonomic units (OTUs) with 97% of identity. The results of bacteria counts were analyzed by Kruskal-Wallis and Mann-Whitney pairwise with $P < 0.05$ , which was considered to indicate a significant difference among groups. Additionally, we used Non-Metric Multidimensional scaling (NMDS) based on count-distance metrics (Bray-Curtis similarity index; ANOSIM:$p < 0.05$) considered to be significant similar between groups using Past3 software (Hammer, Harper, & Ryan, 2001). 8,383,167 raw sequences were obtained from 47 samples, and 6,328,091 high-quality sequences were used for bioinformatics analyses, with an average of 74031± 28677 reads per sample.

### 3.4 Results and Discussion

Dietary high molecular weight of MRPs are largely insoluble in water. They are indigestible molecules that remain in the gastrointestinal tract, which is the major site for biological activities. To illustrate, melanoidins bind to dietary metals, thereby, leading to antioxidant and antimicrobial properties (Morales, Somoza, & Fogliano, 2012). Melanoidin molecules have been shown to suppress *Helicobacter pylori* infection *in vitro* and *in vivo* studies (Hiramoto et al., 2004). The results of studies carried out on MRPs remain controversial impact on health due to the fact that bioconversion of these digestible molecules by the gut microbiota (Hellwig et al., 2015; Seiquer, Rubio, Jesus Peinado, Delgado-Andrade, & Pilar Navarro, 2014). Gut microbiota have been shown to use bread melanoidins as a carbon and nitrogen source and had bifidogenic effects *in vitro* study (Borrelli & Fogliano, 2005), while another *in vitro* study showed the inhibition of enterobacteria (Helou et al., 2015). The objective of this study was to determine the effect of a bread melanoidin model on the composition of rat's gut microbiota and potential positive modulation of the microbiota profile associated with experimental colitis.

3.4.1 Impact of TNBS treatment on the gut microbiota

There were slight differences (not statistically significant) at all taxonomic levels between control and TNBS groups (Figure 3.1A). At the phylum level, the relative abundances of Firmicutes were lower in the TNBS group, compared to the control (Figure 3.2; $P<0.05$). However, there were no significant differences in the relative abundances of Bacteroidetes, Proteobacteria, Actinobacteria, and Verrucomicrobia between control and TNBS groups (Figure 3.2). There were no significant differences between TNBS and control groups of the relative abundance of several Bacteroidetes genera (*Parabacteroides, Bacteroides,* and *Prevotella*) (Figure 3.3), as well as, the Enterobacteriaceae (Proteobacteria) (Figure 3.4), *Bifidobacterium* (Actinobacteria), *Akkermansia* (Verrucomicrobia) (Figure 3.5), and several Firmicutes genera (*Oscillibacter, Faecailibacterium, Blautia*, *ClostridiumXIVa*, *Dorea*, *Lactobacillus*, and *ClostridiumXI*) (Figure 3.6 A, B, C).

However, the relative abundances of Lachnospiraceae (Firmicutes) were lower in the TNBS group, compared to the control (Figure 3.4; 3.6 B; $P<0.05$). In a previous study, TNBS resulted in decreases of Firmicutes and Lachnospiraceae (ALJahdali et al., 2017). However, this study and many others (Ettreiki et al., 2012) also reported that Proteobacteria, specifically Enterobacteriaceae bloom was the most notable impact of TNBS treatment. The absence of such a bloom in our study suggests that the TNBS treatment was not as strong in inducing colitis and dysbiosis as expected. Indeed, low-grade inflammation was observed, with no weight loss or changes in feed intake, but significant alteration of inflammation markers (Data not shown).

3.4.2 Impact of MFC on the rats gut microbiota

By using the NMDS plots, slight differences (not statistically significant) were observed at all taxonomic levels between MFC and control groups (Figure 3.1B). At the phylum level, there were no significant differences of Firmicutes, Bacteroidetes, Proteobacteria, and Verrucomicrobia between control and MFC, but a significant increase of Actinobacteria was observed in MFC, compared to the control (Figure 3.2B; $P > 0.05$). The current findings are in line with the previous report that demonstrates no impact of MFC (low on melanoidins) on Firmicutes, Bacteroidetes and lactic flora using a real time quantitative polymerase chain reaction and cultivation approaches in an *in vivo* study (Helou et al., 2017). However, this study found that the relative abundance of Actinobacteria was higher in MFC, compared to the control, which may be in line with the previous report of increases in the proportion of the population of *Bifidobacteria* (Actinobacteria) in rats that consumed whey-fortified breads (low on melanoidins) (Wronkowska et al., 2017).

There were no significant differences among MFC and control groups in the relative abundance of several Firmicutes genera (*Oscillibacter*, *Faecailibacterium*, *Blautia*, *ClostridiumXIVa*, *Dorea*, and *ClostridiumXI*) (Figure 3.6 A, B, C), as well as, *Akkermansia* (Verrucomicrobia; Figure 3.5), and *Prevotella* (Bacteroidetes; Figure 3.3). Significant decreases of *Parabacteroides* and *Bacteroides* were observed in MFC group, compared to the control (Figure 3.3; $P<0.05$). Decreased proportions of *Parabacteroides* and *Bacteroides* were also seen in rumen microbiome of buffaloes fed wheat and maize grain (Kala et al., 2017). However, increases the proportions of the relative abundances of *Bacteroides* and *Parabacteroides* were found in mice fed high proteins and low carbohydrates (Kim, Kim, & Park, 2016). Also, a significant decrease of Enterobacteriaceae (Proteobacteria) was observed in rats that were fed MFC, compared to the control (Figure 3.4; $P<0.05$). The current findings are in line with the previous report that the

relative abundance of enterobacteria decreased in individuals who consumed bread (Helou et al., 2015).

However, the consumption of MFC resulted in a significant increase of *Lactobacillus*, compared to the control (Figure 3.6 C; $P>0.05$), which may be in line with the previous report of increases in the proportion of the intestinal *Lactobacillus* spp. after adding pre-melanoidins products (low on melanoidins) in batch cultures (Jemmali, 1969). The relative abundances of *Bifidobacterium* (Actinobacteria) were significantly different the in the MFC group, compared to the control (Figure 3.5; $P>0.05$). A significant increase of *Bifidobacterium* was observed in mice fed a MFC diet (Helou et al., 2017).

By using the NMDS plots, there were slight differences between TNBS and MFC-TNBS group at all taxonomic levels (Figure 3.1C). At the phylum level, the relative abundances of Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria showed no significant differences between TNBS and MFC-TNBS groups (Figure 3.2). However, there were significant differences in the relative abundance of Verrucomicrobia between TNBS and MFC-TNBS groups (Figure 3.2; $P>0.05$). There were no significant differences of Firmicutes, Proteobacteria, Verrucomicrobia, and Actinobacteria between MFC and MFC-TNBS groups (Figure 3.2). However, the relative abundances of Bacteroidetes were significantly different in the MFC-TNBS group, compared to MFC group (Figure 3.2; $P>0.05$).

There were no significant differences among TNBS and MFC-TNBS groups in the relative abundances of several Bacteroidetes genera (*Parabacteroides*, *Bacteroides*, and *Prevotella*) (Figure 3.3), as well as, Enterobacteriaceae (Proteobacteria; Figure 3.4), and several Firmicutes genera (*Faecailibacterium*, *Blautia*, *ClostridiumXIVa*, *Dorea*, and *ClostridiumXI*) (Figure 3.6 A, B, C). However, the relative abundances of *Akkermansia* (Verrucomicrobia) were significantly

lower in the MFC-TNBS group, compared to the TNBS group (Figure 3.5; *P*>0.05). *Akkermansia muciniphila* had beneficial effects on the immune system by alleviating inflammation (Wu et al., 2017). The high abundance of *Akkermansia muciniphila* was found in humanized rats fed inulin and arabinoxylans, which are important ingratiates in baked products (Van den Abbeele et al., 2011).

The abundance of *Oscillibacter* and *Lactobacillus* (Firmicutes Figure 3.6A C; *P*>0.05) as well as *Bifidobacterium* (Actinobacteria; Figure 3.5; *P*>0.05) were higher in the MFC-TNBS group, compared to TNBS group. There were no significant differences between MFC and MFC-TNBS groups in the relative abundance of *Prevotella* (Bacteroidetes; Figure 3.3), Enterobacteriaceae (Proteobacteria; Figure 3.4), *Akkermansia* (Verrucomicrobia; Figure 3.5), *Bifidobacterium* (Actinobacteria; Figure 3.5), and several genera of Firmicute (*Oscillibacter*, *Faecailibacterium*, *ClostridiumXIVa*, *Dorea*, *Lactobacillus* and *ClostridiumXI*) (Figure 3.6 A B C). However, significant increases in *Parabacteroides* and *Bacteroides* (Bacteroidetes) were present in the MFC-TNBS group, compared to the MFC group (Figure 3.3 *P*>0.05). Also, the relative abundances of Ruminococcaceae and *Blautia* (Firmicutes) were increased significantly in the MFC-TNBS group, compared to the MFC group (Figure 3.6A B *P*>0.05).

3.4.3 Impact of BCM on the rat's gut microbiota

By using the NMDS plots, there were significant differences between control and BCM groups at all taxonomic levels (Figure 3.1B; ANOSIM *P*<0.05). At the phylum level, the consumption of BCM resulted in a significant increase of Firmicutes, compared to the control (Figure 3.2; *P*<0.05). However, there were decreased abundances of Bacteroidetes and Verrucomicrobia, compared to the control group (Figure 3.2; *P*<0.05). There were no significant differences in the relative abundances of Proteobacteria and Actinobacteria between BCM and

control groups (Figure 3.2). A decrease of Bacteroidetes was detected in individuals who consumed refined wheat breads (Lappi et al., 2013). In contrast, there was no impact of phyla level in mice that consumed BCM, compared to the control group (Helou et al., 2017).

The relative abundance of *Parabacteroides* (Bacteroidetes; Figure 3), *Bifidobacterium* (Actinobacteria; Figure 3.5), and several Firmicutes genera (*ClostridiumXIVa*, *Dorea*, and *ClostridiumXI*) were not significantly different between BCM and control groups (Figure 3.6A B C). BCM consumption resulted in significant increase of Ruminococcaceae, *Oscillibacter, Faecalibacterium*, Lachnospiraceae, and *Blautia* (Firmicutes; Figure 3.6A B $P>0.05$), which may be in line with a previous study of an increase of *Oscillibacter* found in individuals who consumed diets high in resistant starch (RS) and carbohydrate diets (Walker et al., 2011). *Faecalibacterium prausnitzii* is important commensal bacterium produced butyrate and known for their ability of anti-inflammatory effects in the gut (Sokol et al., 2008).

However, there were significant decreases of the relative abundance of *Prevotella* and *Bacteroides* (Bacteroidetes; Figure 3.3 $P<0.05$), as well as, Enterobacteriaceae (Proteobacteria; Figure 3.4 $P<0.05$), *Akkermansia* (Verrucomicrobia; Figure 3.5 $P<0.05$) and *Lactobacillus* (Firmicutes; Figure 3.6 C $P<0.05$) in the group that consumed BCM, compared to the control group. In the same line to our results, a limited growth of Enterobacteriacea and *Bacteroides* spp. was present after 10 hours of fermentation on bread crust melanoidins in an *in vitro* study (Borrelli & Fogliano, 2005). There were no significant differences between Enterobacteriacea and *Bacteroides* spp in mice that consumed bread crust, compared to a control group (Delgado-Andrade et al., 2017). However, there was significant decrease of *Lactobacillus* spp. in mice that were fed bread crust diet, compared to a control group (Delgado-Andrade et al., 2017). Also, *Lactobacillus* spp. showed a low ability to use bread melanoidins for their growth (Borrelli &

Fogliano, 2005). Species related to *Bacteroides* and *Prevotella* decreased in individuals who consumed refined wheat breads (Lappi et al., 2013). On the other hand, another study on the consumption of coffee melanoidins resulted in increases of species belonging to Bacteroides-Prevotella in an *in vitro* study (Gniechwitz, Reichardt, Blaut, Steinhart, & Bunzel, 2007).

By using the NMDS plots, there were significant differences between TNBS and BCM-TNBS groups at all taxonomic levels (Figure 3.1C; ANOSIM $P<0.05$). At the phylum level, the relative abundances of Firmicutes and Verrucomicrobia showed significant differences between TNBS and BCM-TNBS groups (Figure 3.2; $P<0.05$). However, there were no significant differences of Bacteroidetes, Proteobacteria, and Actinobacteria between the BCM-TNBS group and the TNBS group (Figure 3.2). There were no significant differences of Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, and Actinobacteria between BCM and BCM-TNBS groups (Figure 3.2). Highly heated food rich in Maillard reaction products protected against inflammation of experimental colitis in mice (Anton, Craus, Niquet-Leridon, & Tessier, 2012). We showed that consumption of BCM had to limit the gut microbiota dysbiosis in BCM-TNBS groups. Nε-carboxymethyllysine, an advanced Maillard reaction products, alleviates gut microbiota dysbiosis in colitic mice (ALJahdali et al., 2017).

There were no significant differences between TNBS and BCM-TNBS groups in the relative abundances of several Bacteroidetes genera (*Parabacteroides*, *Bacteroides*, and *Prevotella*) (Figure 3.3), as well as, *Bifidobacterium* (Actinobacteria; Figure 5), and several Firmicutes genera (*Oscillibacter*, *Faecailibacterium*, *Blautia*, *Dorea*, *Lactobacillus* and *ClostridiumXI*) (Figure 3.6 A, B, C). The relative abundances of Enterobacteriaceae (Proteobacteria; Figure 3.4 $P<0.05$) and *Akkermansia* (Verrucomicrobia; Figure 3.5 $P<0.05$) were significantly lower in the BCM-TNBS group, compared to the TNBS group. Nε-

carboxymethyllysine, an advanced Maillard reaction products, limited Enterobacteriaceae dysbiosis in colitic mice (ALJahdali et al., 2017). *Akkermansia* spp. are known as mucin-degrading bacteria that consumed glycated proteins as an energy source (Tailford, Crost, Kavanaugh, & Juge, 2015). *Akkermansia muciniphila* is known to possess anti-inflammatory effects. However, the abundances of Lachnospiraceae (Firmicutes) and *Clostridium XIVa* were higher in the BCM-TNBS group, compared to the TNBS group (Figure 3.6 B; $P < 0.05$).

There were no significant differences between BCM and BCM-TNBS groups in the relative abundance of *Parabacteroides* (Bacteroidetes; Figure 3.3), Enterobacteriaceae (Proteobacteria; Figure 3.4), *Akkermansia* (Verrucomicrobia; Figure 3.5), *Bifidobacterium* (Actinobacteria; Figure 3.5), and several genera of Firmicute (*Blautia*, *ClostridiumXIVa*, and *Dorea*) (Figure 3.6 B). Significant increases of *Prevotella* and *Bacteroides* (Bacteroidetes) were present in the BCM-TNBS group, compared to the BCM group (Figure 3.3 $P>0.05$). Also, the relative abundances of Ruminococcaceae and several genera of Firmicutes (*Oscillibacter* and *Faecailibacterium*) were significantly higher in the BCM group, compared to the BCM-TNBS group (Figure 3.6A $P>0.05$). Conversely, the relative abundances of *ClostridiumXIVa*, *Lactobacillus* and *ClostridiumXI* were significantly higher in BCM-TNBS group, compared to the BCM group (Figure 3.6 B&C $P >0.05$).

**3.5 Conclusions**

We concluded that MFC increased the relative abundances of *Bifidobacterium* spp. and *Lactobacillus* spp., while BCM increased beneficial microorganisms *Faecalibacterium* spp. In addition, both MFC and crust had to limit increases of Enterobacteriaceae. The BCM -TNBS group might be able to limit gut microbiota dysbiosis in colitic mice.

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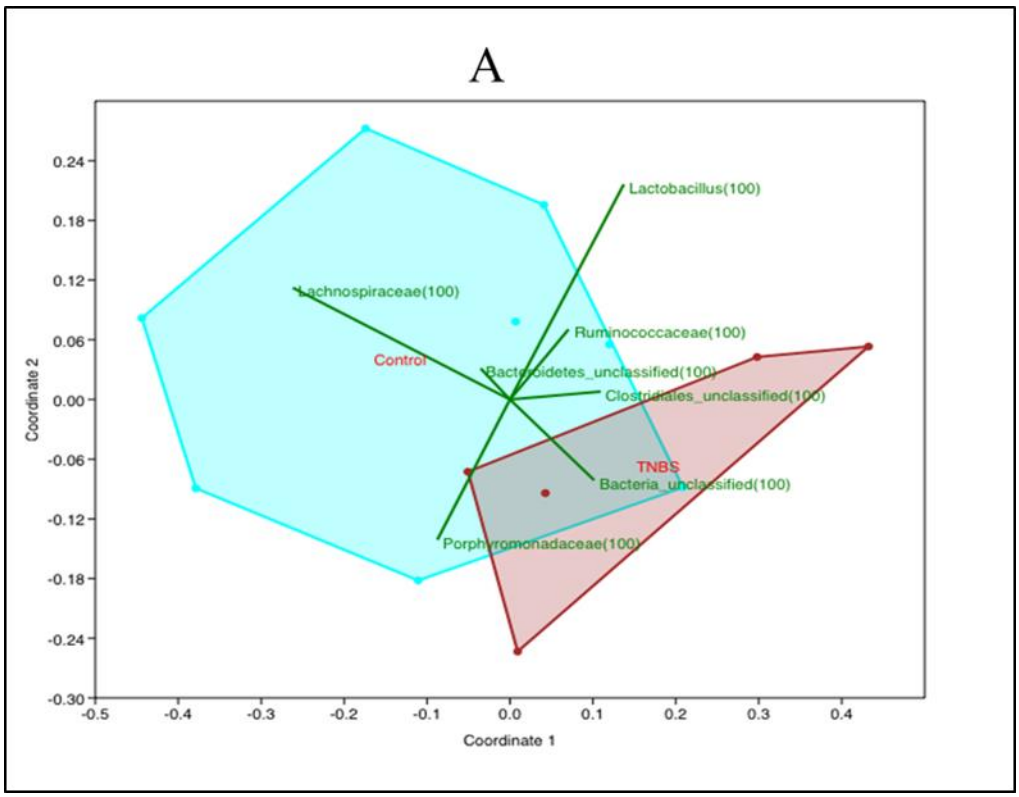
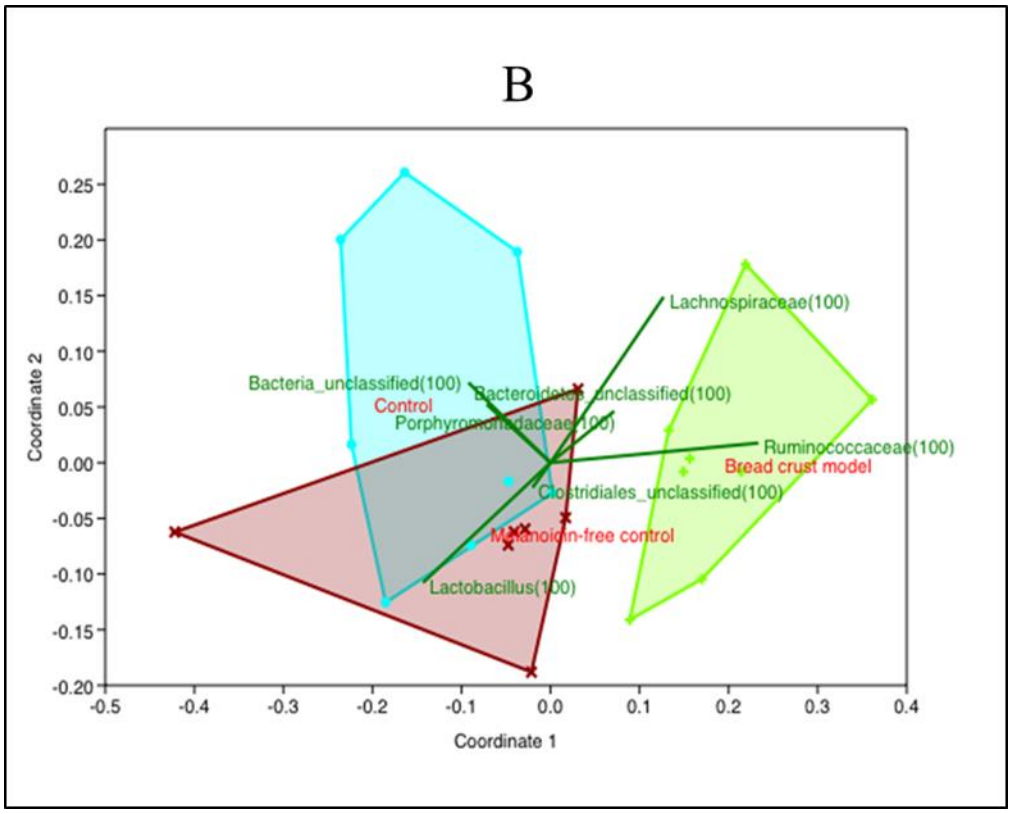

A
Lactobacillus(100)
Lachnospiraceae(100)
Ruminococcaceae(100)
Control
Bacteroidetes_unclassified(100)
Clostridiales_unclassified(100)
TNBS
Bacteria_unclassified(100)
Porphyromonadaceae(100)
Coordinate 2
Coordinate 1


B
Lachnospiraceae(100)
Bacteria_unclassified(100)
Control
Porphyromonadaceae(100)
Ruminococcaceae(100)
Bread crust model
Clostridiales_unclassified(100)
Melanoidin-free control
Lactobacillus(100)
Coordinate 2
Coordinate 1

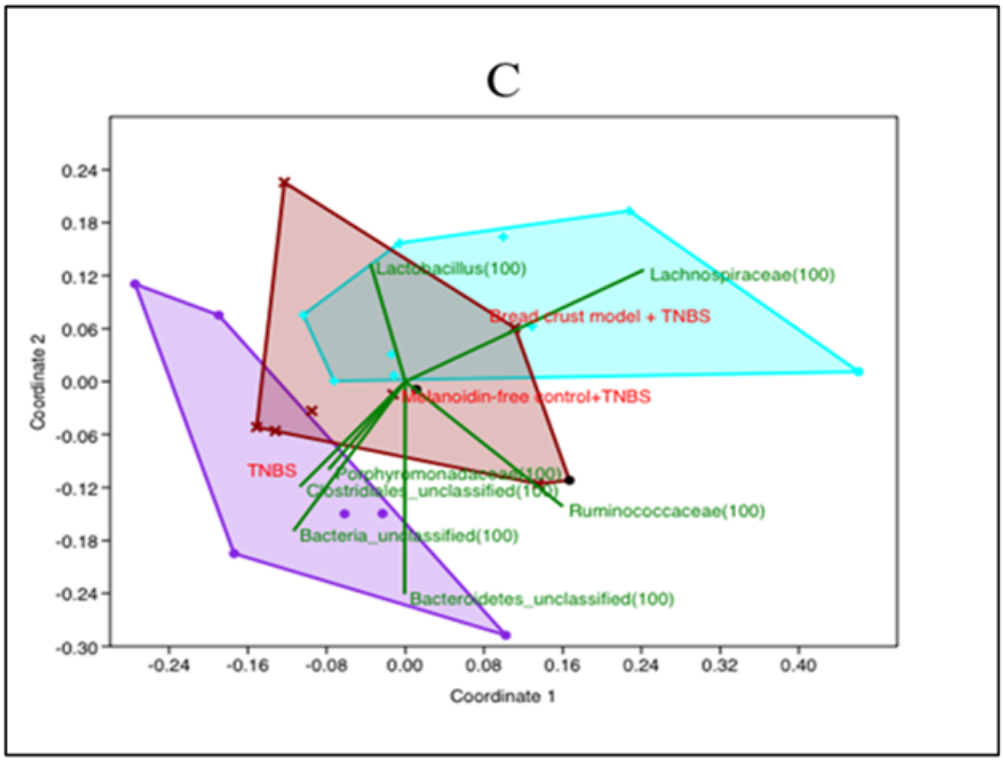
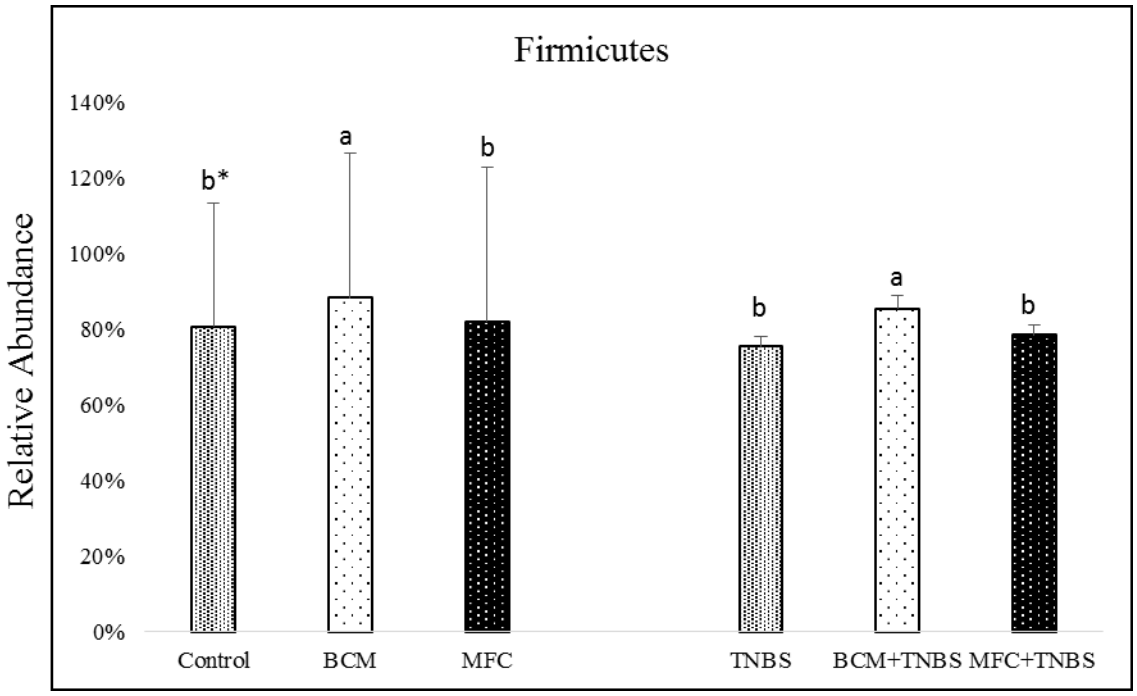
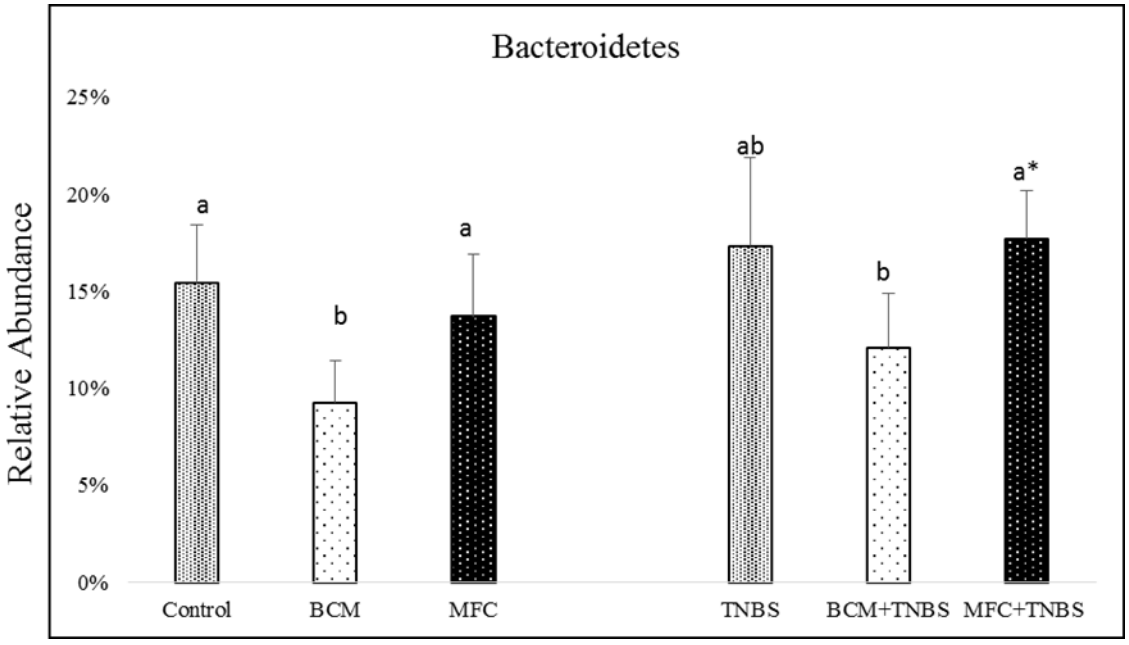
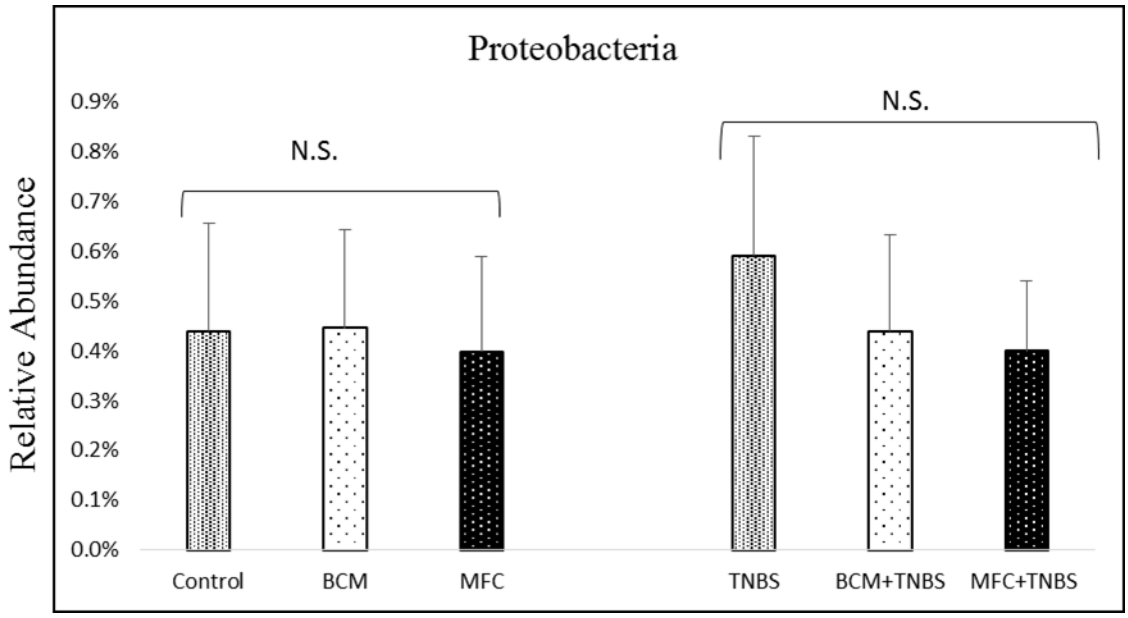
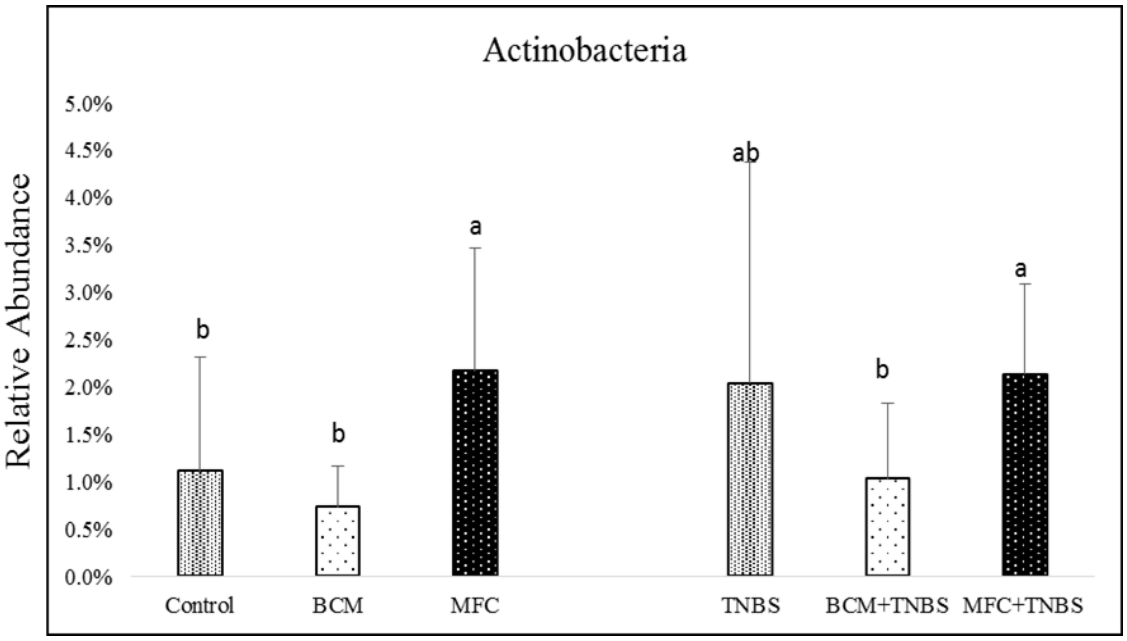



**Figure 3.1:** Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of the healthy and TNBS-treated rats (NMDS plot). A slight difference was observed between TNBS and control (A). No significant differences showed between control and melanoidin-free control, but significant differences were observed between control and bread crust model (ANOSIM $P$>0.05) (B). Slight significant differences showed between TNBS and melanoidin-free control in TNBS-treated rats, but significant differences were observed between TNBS and bread crust model in TNBS-treated rats (C) (ANOSIM $P$ >0.05).


Firmicutes
Relative Abundance
140%
120%
100%
80%
60%
40%
20%
0%
b*
a
b
b
a
b
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS


Bacteroidetes
Relative Abundance
25%
20%
15%
10%
5%
0%
a
b
a
ab
b
a*
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS


Proteobacteria
Relative Abundance
0.9%
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
N.S.
N.S.
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS


Actinobacteria
Relative Abundance
5.0%
4.5%
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
b
b
a
ab
b
a
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS

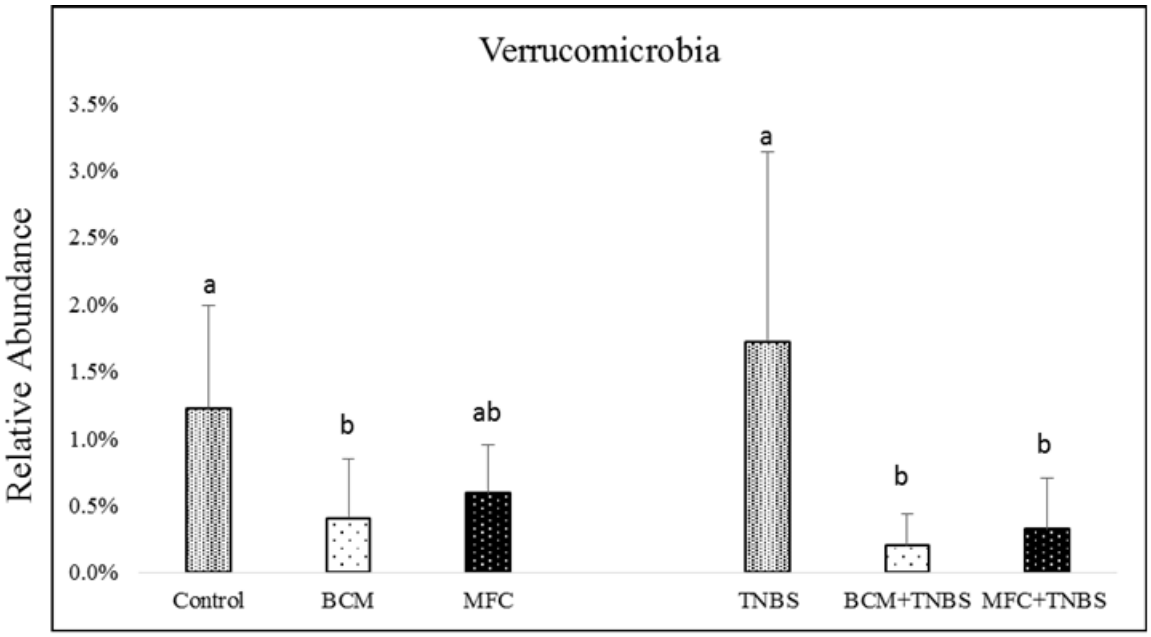
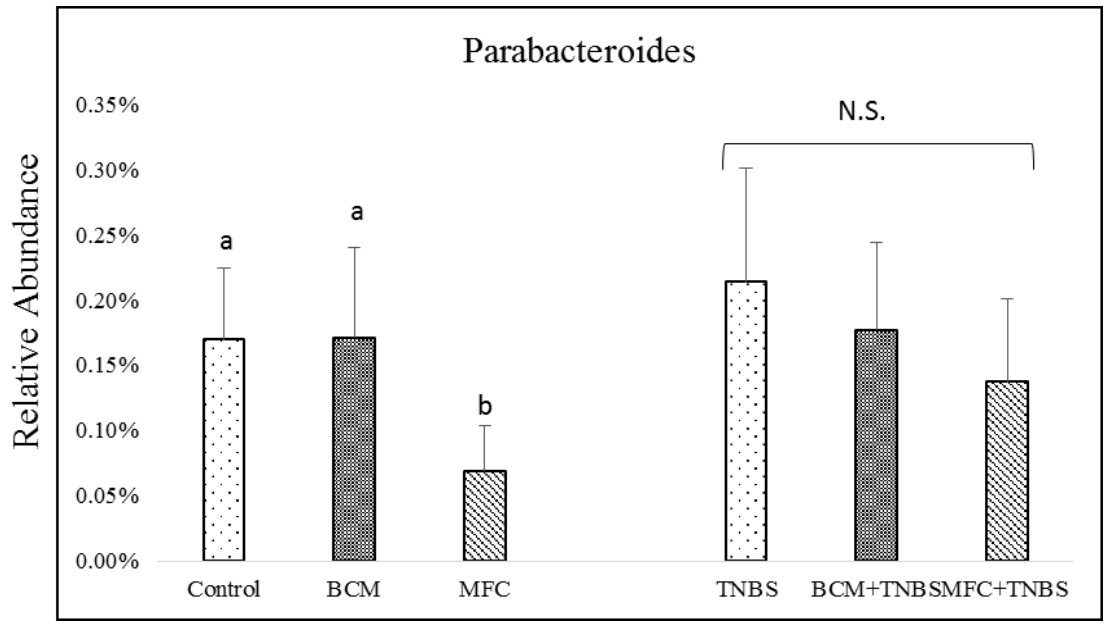
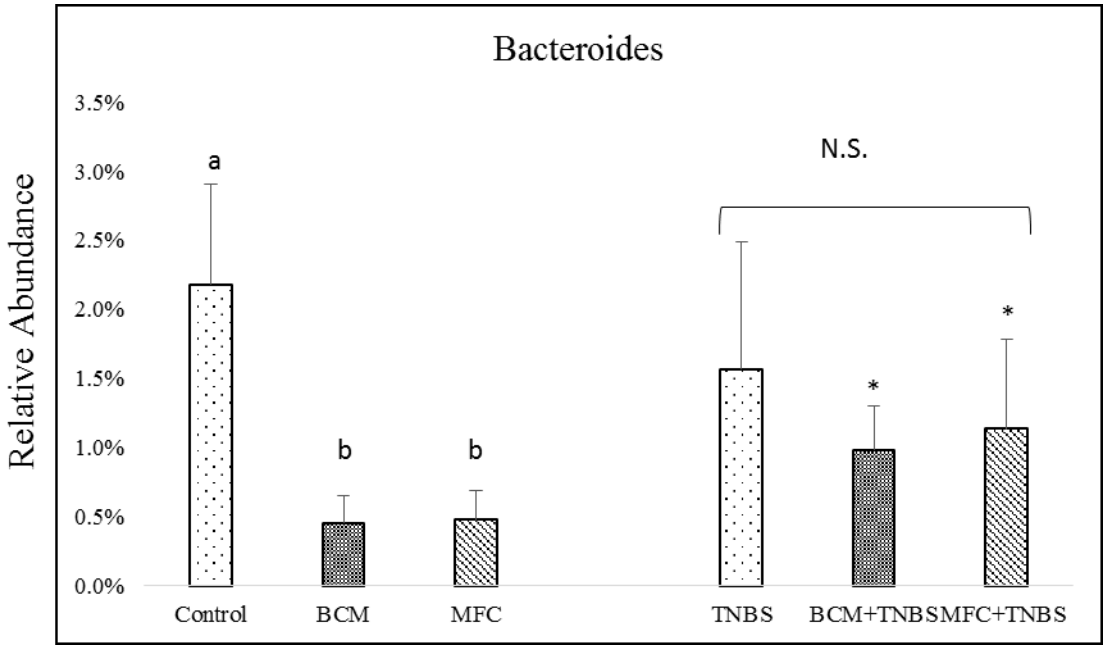



**Figure 3.2**: Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy and TNBS-treated rats in Phyla level. The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.


Parabacteroides
Relative Abundance
0.35%
0.30%
0.25%
0.20%
0.15%
0.10%
0.05%
0.00%
a
a
b
N.S.
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS


Bacteroides
Relative Abundance
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
a
b
b
N.S.
*
*
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS

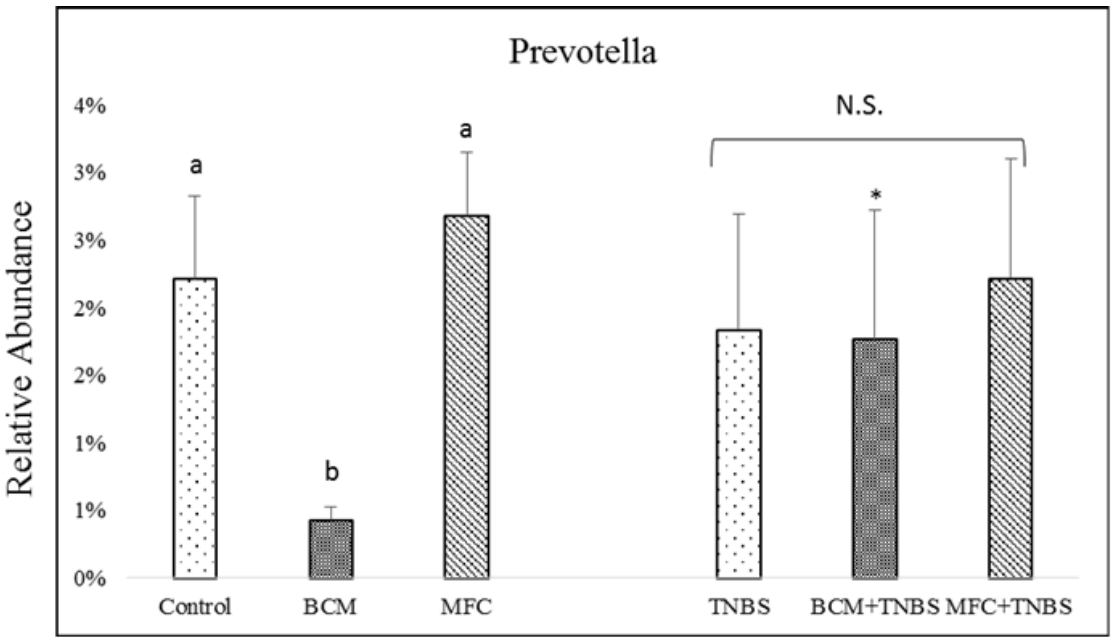



**Figure 3.3:** Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy and TNBS-treated rats in genera of Bacteroidetes. The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.

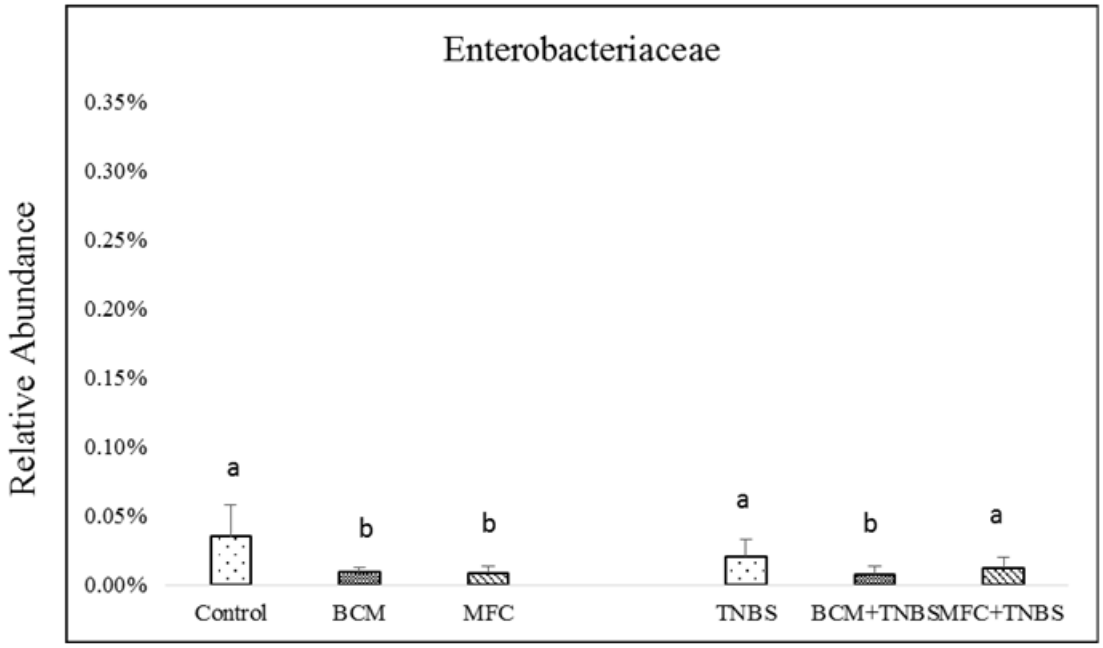



**Figure 3.4:** Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy and TNBS-treated rats in family of Proteobacteria (Enterobacteriaceae). The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.

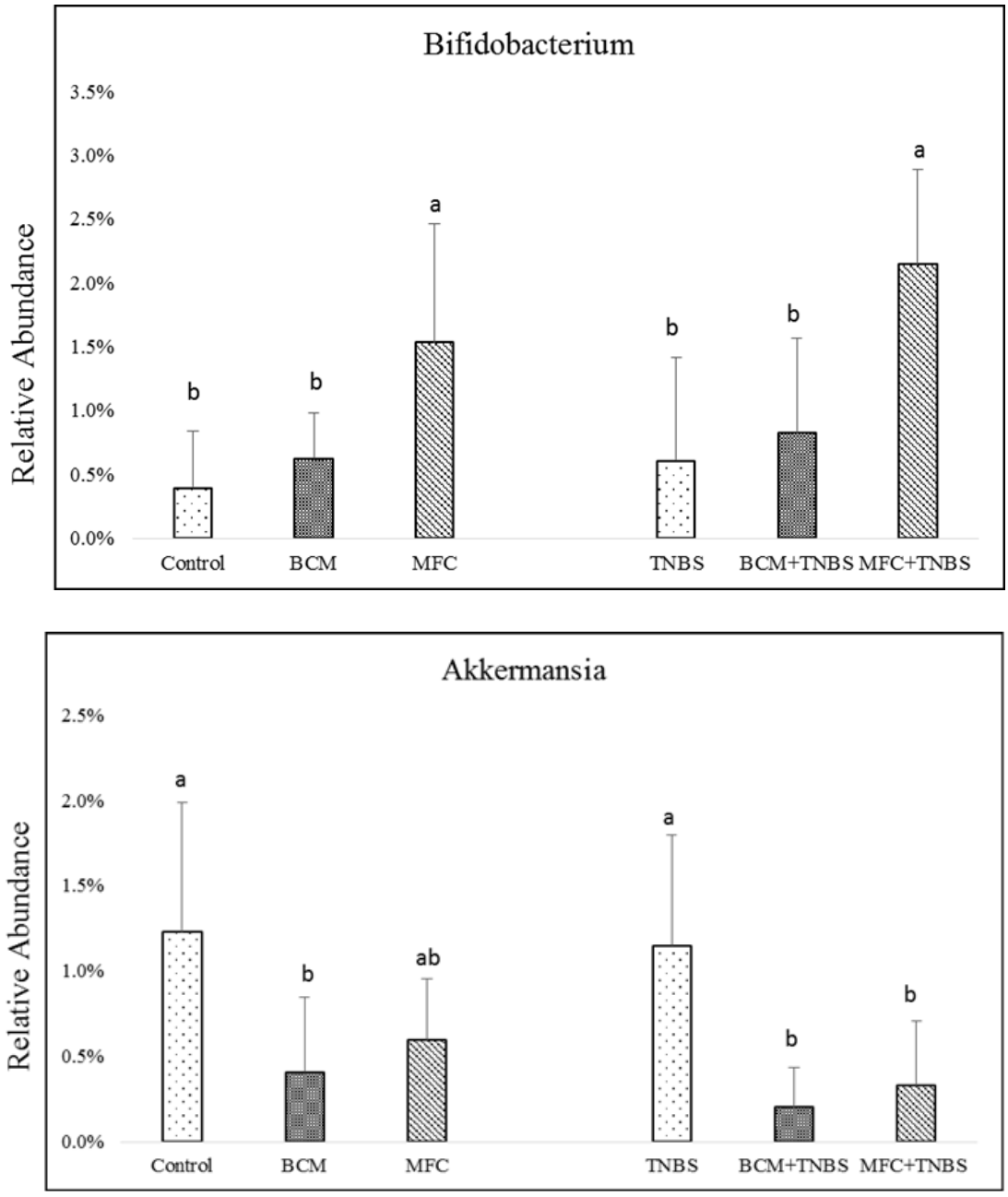
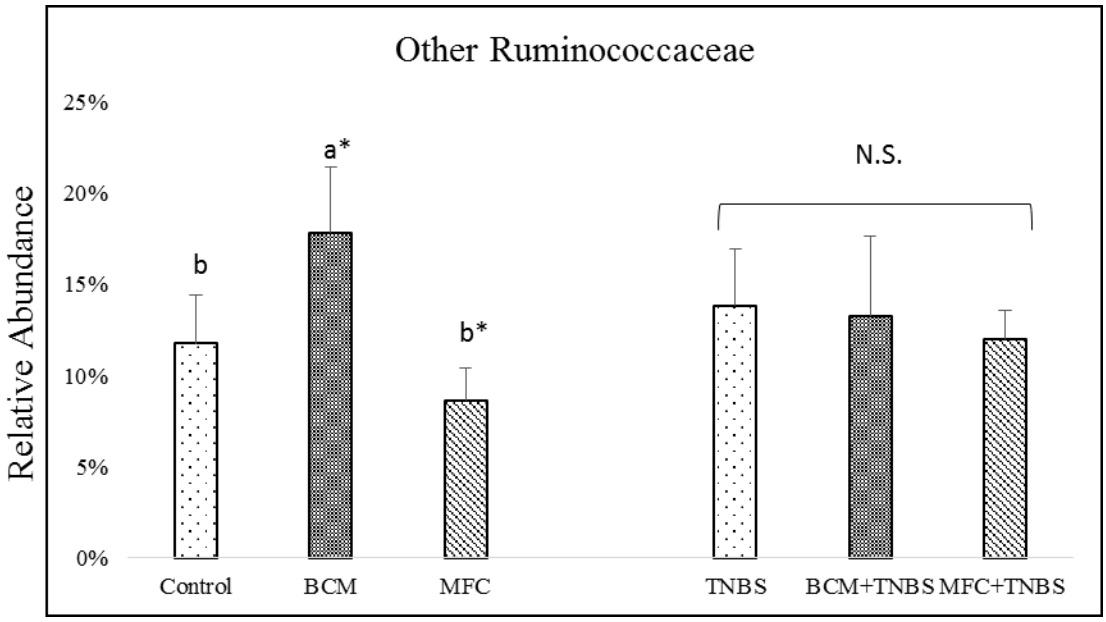
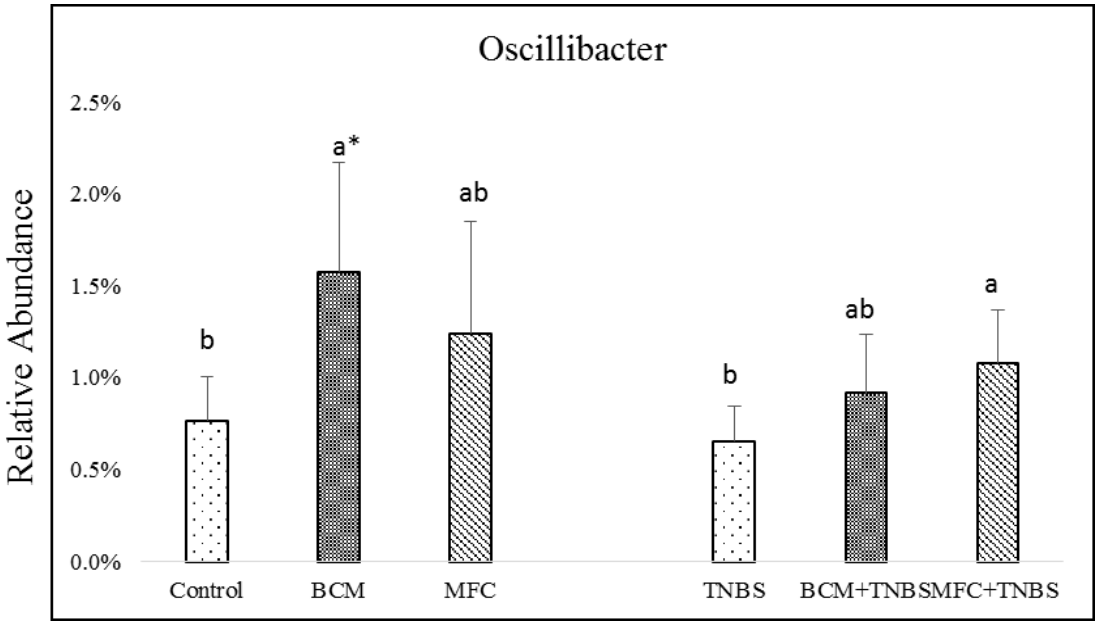



**Figure 3.5:** Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy and TNBS-treated rats in genera of Actinobacteria and Verrucomicrobia. The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.


Other Ruminococcaceae
Relative Abundance
25%
20%
15%
10%
5%
0%
a*
b
b*
N.S.
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS


Oscillibacter
Relative Abundance
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
a*
ab
b
b
ab
a
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS

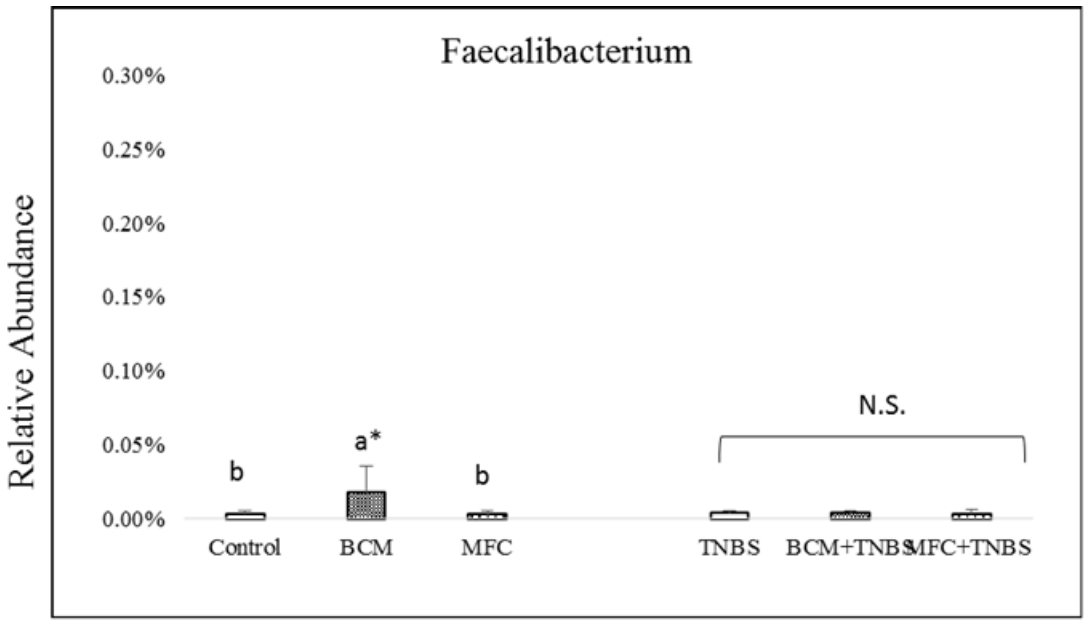
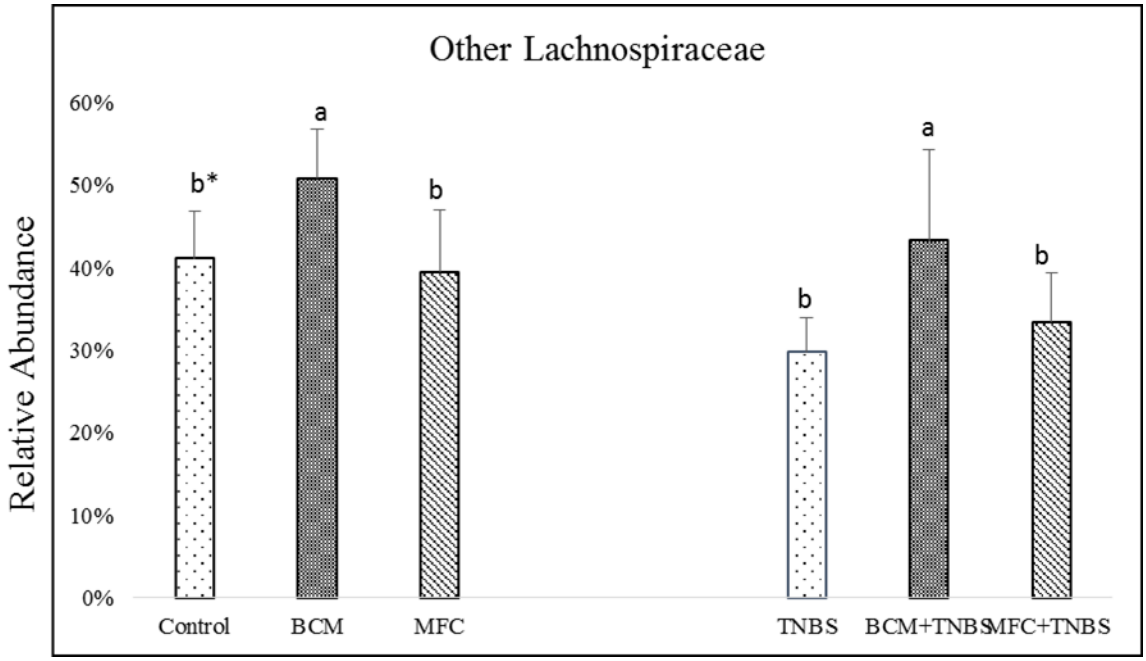
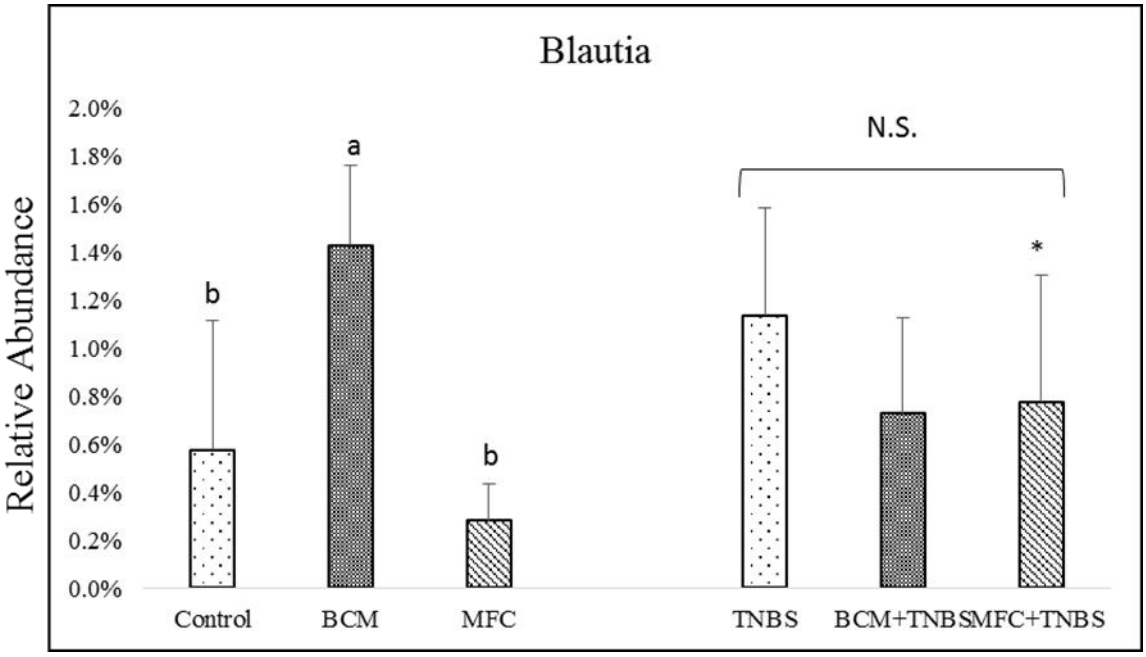



**Figure 3.6 (A):** Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy and TNBS-treated rats in genera of Firmicutes. The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.


Other Lachnospiraceae
Relative Abundance
60%
50%
40%
30%
20%
10%
0%
b*
a
b
b
a
b
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS


Blautia
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
b
a
b
N.S.
*
Control
BCM
MFC
TNBS
BCM+TNBS
MFC+TNBS

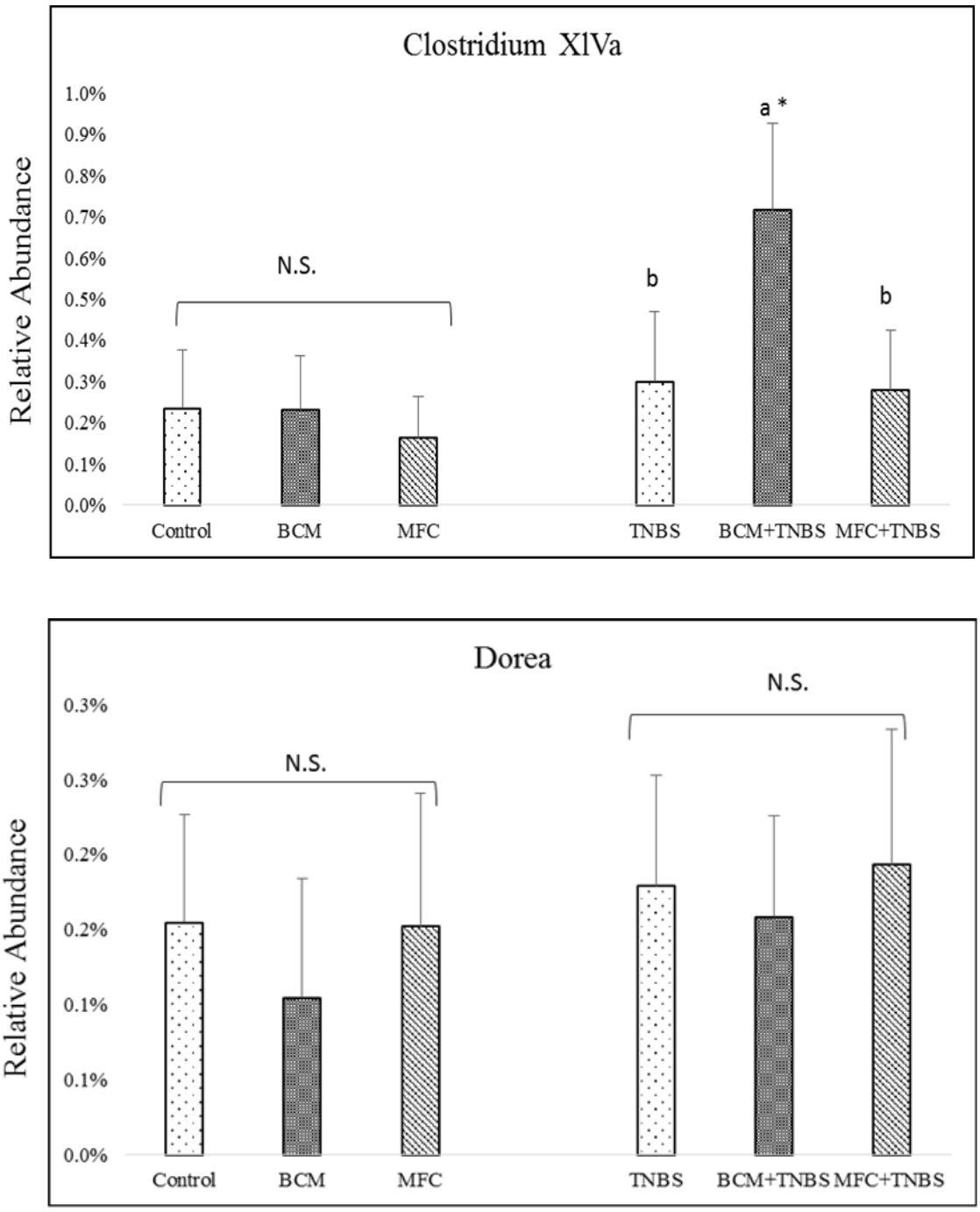



**Figure 3.6 (B):** Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy and TNBS-treated rats in genera of Firmicutes. The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.

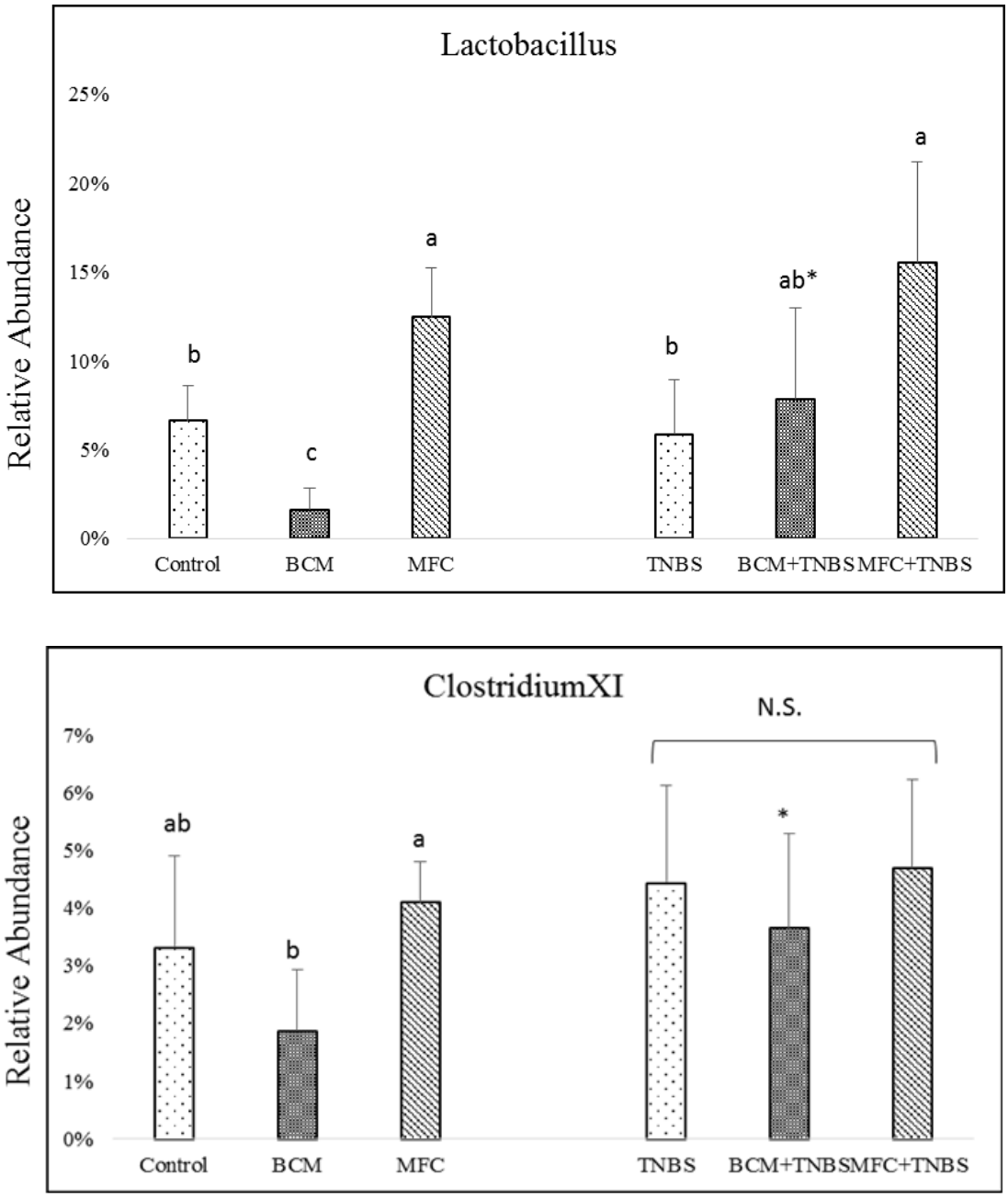



**Figure 3.6 (C)**: Impact of bread crust model (BCM) and melanoidin-free control (MFC) on the gut microbiota of healthy rats and TNBS-treated mice in genera of Firmicutes. The letters indicated significant differences ($P$<0.05) between diets. The symbol indicated significant differences ($P$<0.05) between treatments.

CHAPTER FOUR

RESEARCH ARTICLE

# Modulation of Mice Gut Microbiota by Increasing Amounts of Dietary melanoidins from barley malts

ALJahdali Nesreen [1*] and Carbonero Franck [1,2*]

[1]Cell and Molecular Biology Program; University of Arkansas, AR, USA

[2]Department of Food Science; University of Arkansas, AR, USA

[*]Corresponding author:

Franck Carbonero

Department of Food Science

University of Arkansas

2650 Young Avenue

Fayetteville, AR 72704

(+1) 479-575-6822

Email: fgcarbon@uark.edu

**4.1 Abstract:**

Melanoidins are the final Maillard reaction products produced in food by prolonged and intense heating, and are protein-carbohydrate complexes with high molecular weights. Dietary melanoidins from bread crust and coffee have been reported as harmless and even potentially beneficial. Here, we assessed the impact of consumption of increasing amounts of malt melanoidins on mice gut microbiota, another dietary source that has not been studied so far. Seventy five mice were divided equally into 5 groups, the control group consumed a diet with 0% of melanoidin-rich malts, and other groups received melanoidin malts in increments of 25%, up to 100% melanoidin malts. Feces were sampled at days 0, 1, 2, 3, 7, 14 and 21, and microbial DNA was extracted from fecal sample. Microbiota was determined V4 bacterial 16S rRNA amplicon sequencing, and short-chain fatty acids (SCFA) by Gas Chromatography. The consumption of malts resulted in a significant decrease of Firmicutes and a distinctive increase of Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria during the study. Increased melanoidins was found to result in significantly divergent gut microbiota profiles all along the study, as well as to maintain SCFA production to the baseline levels. The relative abundance of *Dorea*, *Oscillibacter*, and *Alisitpes* were decreased, but the relative abundance of *Lactobacillus*, *Parasutterella*, *Akkermansia*, *Bifidobacterium*, and *Barnesilla* were increased throughout the study. *Bifidobacterium* spp. and *Akkermansia* spp. were significantly increased in mice consuming the highest melanoidins amounts. The results support the hypothesis that malt melanoidins have remarkable prebiotic properties that may be valorized in food development.

**Keywords:** Maillard reaction products, Melanoidins, Short-chain fatty acids, Gut microbiota, Prebiotic

## 4.2 Introduction:

The Maillard reaction (MR) generates several low weight molecules from reducing sugars and amino acids during food thermal processing and storage, such as Amadori rearrangement products, furfural, reductones, and other dicarbonyl compounds. These low weight compounds are often recombined through a range of advanced MR to form Melanoidins [1], which are the final products of the MR [2]. Melanoidins are brownish, heterogonous, insoluble molecules, and were traditionally considered as high molecular weight (HMW) molecules [3,4], but recent reports have shown that melanoidins also include a low molecular weight (LMW) fraction [5]. Melanoidins produced in foods are predominantly HMW, and melanoidins is molecular weight that is directly correlated with heating intensity and time [6]. For example, the average molecular weight (MW) of unroasted malts were <10 Kilodalton (kDa), whereas the roasted malts were around 320 kDa [7]. The chemical structures of melanoidins are complex and difficult to determine, but the concentration of sugars and amino acids of roasted barley have been identified [8]. During the roasting of barley, significant increases of total sugar, dextrin, and melanoidins were detected, while hemicellulose and starch significantly decreased [8].

In contrast with other MRPs, melanoidins are generally considered harmless, and even potentially beneficial to human health [9]. Though some studies reported that dietary melanoidins might display moderate genotoxicity and cytotoxicity effects [10,11], several studies reported potential health benefits including antioxidant, antihypertensive, antimicrobial, and prebiotic properties of food melanoidins [12,13]. Data from metabolic transit studies showed that melanoidins can escape digestion and pass into the upper gastrointestinal tract (GIT), where they are likely subject to fermentation by resident gut microbes [14,15]. To illustrate, only 27% of the LMW

constituents of melanoidin products were absorbed in the intestine; and only 4.3% of the HMW melanoidins were excreted in feces and urine [16].

It has actually been suggested that dietary melanoidins may possess prebiotic properties [6], due to their structural similarities with fibers[17]. Interestingly, studies on the interaction between gut microbiota and melanoidins have initially focused on antimicrobial activity, predominantly in batch cultures [18-20]. For instance, data from *in vitro* and *in vivo* studies showed that melanoidins could suppress *Helicobacter pylori* infection [21]. Moreover, melanoidins were shown to kill *Escherichia coli* by causing irreversible changes in both the inner and outer membranes [22]. While knowledge on the role of the gut microbiota in non-digestible polysaccharides and fiber fermentation has become extensive [23,24], gut microbiota fermentation of MRPs has been scarcely studied [25], with even less knowledge on melanoidins [26]. In an *in vitro* study, melanoidins were shown to increase the growth of gut anaerobes during mixed culture growth [27]. *Bifidobacteria* strains were shown to use bread melanoidins as a carbon source in batch cultures [26], while coffee melanoidins increased the number of anaerobic bacteria belonging to *Bacteroides-prevotella* during fermentation in an *in vitro* study [28]. Another food rich in melanoidins is beer, where melanoidins are present in malts, and HMW melanoidins being are more abundant in kilned malts [29]; and this melanoidins source has never been assessed for its effect on the gut microbiota. The objective of this study was to determine the impact of long-term consumption of increasing melanoidins concentration from barley malts on the gut microbiota and fermentation patterns of healthy mice.

## 4.3 Material and Methods:

### 4.3.1 Experimental Animals:

The animal study was conducted at the Animal House Facility of the University of Arkansas after receiving approval from the Institutional Animal Care and Use Committee (IACUC). Seventy-five male mice (*Mus musculus* strain C57BL/6J) aged 8 weeks (20g) were purchased from Jackson Laboratory (Farmington, USA). Mice were housed in stainless steel cages under controlled temperature (70 °F) and a 12 h light-dark cycle, with free access to water and food. Before dietary intervention, mice were provided with Teklad (standard) 40 g of chow pellets (Envigo, Madison, WI).

### 4.3.2 Experimental Design:

Melanoidin-free and melanoidin-rich barley malts were purchased from Weyermann Company (Northern Brewer, USA). Melanoidin-free malts were considered as normal barley grains pre-germinated to release saccharolytic and amylolytic enzymes for beer brewing purposes and containing low amounts of LMW melanoidins (Briess 2-row Malt). Melanoidin-rich malts were considered as enriched in HMW melanoidins due to intense toasting (Weyermann® Melanoidin) resulting in grains browning and specific organoleptic properties [8,30]. Mice were assigned to receive 40 g of melanoidin malts in the first week. The malts portion was increased by 20g increments each consecutive week [23]. They were divided into groups of five with a total of 15 mice in each group, and each major group was split into five sub-groups of three individuals:

Group (1). Melanoidin-free malts (0% melanoidins) only.

Group (2). 75% of melanoidin-free malts and 25% of melanoidin-rich malts.

Group (3). 50% of melanoidin-free malts and 50% of melanoidin-rich malts

Group (4). 25% of melanoidin-free malts and 75% of melanoidin-rich malts

Group (5). Melanoidin-rich malts (100% melanoidins) only.

Malts were added in stainless cages twice a week, and the unconsumed amounts were measured before adding new malts. Body weight was measured at 7, 18, and 25 days. Mice were transferred from stainless steel cages to metabolic cages (Tecniplast Cdd, 170013) for 6 hours/day in order to collect feces at day 0, 1, 2, 3, 7, 14 and 21. Day 0 samples represented the baseline, with all groups previously fed chow pellets. After that, each group was provided with their specific melanoidin malts amounts, over the 21 days.

4.3.3 Fecal Short Chain Fatty Acids (SCFAs) Quantification:

Short chain fatty acids (SCFAs), specifically acetate, propionate, and butyrate, were measured by gas chromatography (GC) for 0% and 100% groups. Briefly, 1 g of fecal samples were transferred into centrifuge tubes, and 9 ml of distilled water were added. After vortexing and centrifugation, 900 μL of the supernatant were transferred into 2 ml tubes containing 100 μL of buffer containing the internal standard 4-methyl-valeric acid (50mM), and meta-phosphoric acid (50%) and copper sulfate (1.56mg/ml). After vortexing and centrifugation, 1 μL of samples were loaded to GC. SCFAs concentrations were estimated from integration of peak areas in relation with acetate, propionate, and butyrate standards (Sigma-Aldrich, Germany) [31].

4.3.4 DNA Extraction and PCR Amplifications:

Genomic DNA was extracted from mice fecal samples using commercial QIAamp DNA stool Mini Kit (Qiagen, Germany) following the manufacturer's protocol, with addition of bead-

beating at 5 m/s for 60 seconds twice [32]. PCR amplifications were performed in 25 μL reactions with: 1 μL of DNA template, 2 μL of universal primer (8F and 1541R), and 22 μL of KAPA HiFi mastermix (KAPA Biosystems, Wilmington, USA), followed by agarose gel electrophoresis by using 1 μL of SYBR safe DNA Gel Stain (Thermo Fisher Scientific, USA) fluorescent dye to confirm the success of the PCR. PCR index was accomplished by targeting the V4 region of the bacterial 16S rRNA gene [33]. Briefly, PCR dual-indexed strategy was performed in 27 μL reaction components with: 2 μL of DNA template; 2 μL of index primers; and 23 μL of AccuPrime™ Taq DNA Polymerase (Invitrogen, USA) following the manufacture's protocol. Amplifications were performed by initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 sec, primer annealing at 55 °C for 30 sec, and extension at 72 °C for 1 min.

4.3.5 Libraries Preparation and Sequencing:

Illumina MiSeq sequencing was used to study the composition of gut microbiota by targeting the V4 region of the bacterial 16S ribosome RNA gene of each group following the dual-indexed strategy [33]. Normalization of the PCR products was completed to elute short primers, unincorporated dNTPs, enzymes, short-failed PCR products, and salts from PCR reactions using Invitrogen SequalPrep kits following the manufacturer's protocol. Q-PCR was performed with the PerfeCta NGS library quantification kits (Quanta Biosciences, USA) following the manufacturer's protocol. Quality check was also performed on a Tape-Station 2100 (Agilent, USA) to provide the exact size of DNA, which were 394 base pair and 424 base pair.

Libraries were pooled, denatured with NaOH, and diluted to 0.75 nM following recommended Illumina protocols. Pooled denatured libraries were diluted to 6 pm as a final concentration, with the addition of 20 pm of Phix V3. The diluted denatured libraries were loaded on an Illumina MiSeq sequencing cartridge V3-600 cycles, with the addition of 3 sequencing

primers due to the use of different indices as described by Kozich et al (2013). The runs were monitored with Sequence Analysis Viewer with particular emphasis on appropriate cluster density (700-800k/mm2) and quality scores (final >Q30 score of >70%).

4.3.6 Bioinformatics and Statistical Analyses:

FASTQ files were readily demultiplexed by the built-in BaseSpace Sequence Hub program and downloaded from the BaseSpace website. 16S amplicon reads were analyzed by using the MOTHUR software package 1.39.5 following the Illumina SOP [34] (https://www.mothur.org/wiki/MiSeq_SOP). Briefly, sequences were screened and aligned to the Silva database for 16S r RNA gene sequences. Subsequently, Operational Taxonomic Units (OTUs) were picked and assigned to taxonomic groups. Resulting OTUs and taxonomic tables were exported to Excel sheets for basic analysis.

The results of feed intake, average daily gain, and short-chain fatty acids were analyzed by analysis of variance (ANOVA) followed by a Tukey post hoc test with $P$ <0.05 as considered to be significant difference between groups. The results of microbiota counts were analyzed by Kruskal-Wallis and Mann-Whitney pairwise with $P < 0.05$ as considered to be significant difference between groups and by Non-Metric Multidimensional scaling (NMDS) based on count-distance metrics (Bray-Curtis similarity index; ANOSIM: $P < 0.05$) considered to be significant similarities between groups using Past3 software [35].

4.4 **Results:**

4.4.1 Impact of diet on Feed Intake and Average Daily Gain:

Feed intake varied considerably during the study for all mice. A significant increase was observed between 4 and 7 day, followed by a return to the baseline feed intake afterwards.

Intriguingly, feed intake decreased significantly after day 18 (Table 4.1). Dietary treatments had little impact on feed intake; with only slight (but significant) increase of feed intake for the 25% group (0.54±0.02 g/day) relative to groups that consumed 0% and 100% melanoidin malts (0.48±0.02 g/day and 0.44±0.02 g/day).

Average daily weight gain (ADG) varied during the study ($P$<0.05; Table 4.2).The 0% and 25% groups had significantly lower ADG during the first 7 days, and significantly higher ADG in the last 7 days. The 50 and 75% groups had completely inverse dynamics in ADG (Table 4.2). The 100% group maintained relatively low ADG throughout the study.

4.4.2 Consequence of SCFAs of Melanoidin Malts on Healthy Mice:

The quantity of SCFAs measured in feces from 0% and 100% groups are shown in Table 3 from day 0 to day 21. Overall the total SCFAs production decreased significantly in mice that received 0%, but they remained stable in mice fed 100% of melanoidin malts over the experiment. There were no apparent differences in the proportion of acetate in mice fed 0% and 100% at days 0, 3, 7, and 14. However, there were significant differences of acetate in mice that received 0% of melanoidin malts with mice that consumed 100% of melanoidin malts at day 21 ($P$<0.05; Table 4.3) and consumption of melanoidins maintained acetate production at stable levels, while consumptions of non-melanoidin malts resulted in significant decrease. Significant decreases in the quantity of propionate were observed in mice fed 0% at days 14 and 21 and in mice that consumed 100% of melanoidin malts at days 7, 14, and 21. The amount of propionate between 0% and 100% of melanoidin malts was significantly different at day 21 ($P$<0.05; Table 4.3), and also melanoidin-malt consumption allowed for maintenance of higher propionate production. Significant decreases in the amount of butyrate were observed in mice fed 0% at days 14 and 21 and in mice that consumed 100% of melanoidin malts at days 3, 7, 14, and 21. The quantity of

butyrate between 0% and 100% of melanoidin malts was significantly different at day 14 ($P$<0.05; Table 4.3).

4.4.3 Gut microbiota analyses:

From 175 samples, a total of 10,123,928 raw sequences were obtained, of which 9,290,708 high quality reads were used for further analysis. Samples yielding less than 3000 high-quality reads (n=9) were discarded; the remaining samples analyzed had an average 50, 20, 20 ± 37, 097 SE reads per sample. All reads were analyzed together in the MOTHUR1.39.5 pipeline.

*4.4.3.1 Impact of diet on gut microbiota profiles and dynamics:*

The NMDS plots for the five days 0, 3, 7, 14, and 21 showed that the gut microbiota profiles of the five groups became distinctly different at days 3, 7, 14, and 21 (ANOSIM $P$<0.05), while they were not distinguishable on day 0 (Figure 4.1). On day 3, the groups that consumed higher percentage of melanoidin malts (75% and 100%) were significantly different from the other groups (0% and 25%), while the 50% group was not significantly different from any group (Figure 4.1; ANOSIM $P$<0.05). On day 7, the groups that consumed melanoidin malts (25%, 50%, 75%, and 100%) were all significantly different from the group that consumed 0% of melanoidin malts (Figure 4.1; ANOSIM $P$<0.05). On day 14, the 75% and 100% groups clustered significantly apart from the 0% and 25% groups (Figure 4.1; ANOSIM $P$<0.05). Long-term (Day 21) consumption of melanoidins resulted in significant clustering distinguishing the 50%, 75%, and 100% groups on one side, and the 0% and 25% groups on the other side (Figure 4.1; ANOSIM $P$<0.05).

*4.4.3.2 Impact of melanoidin malts on the composition of the gut microbiota:*

Overall, the most abundant phyla detected were Firmicutes, Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria. The consumption of any combination of malts resulted in a

significant decrease of Firmicutes. Conversely, malts consumption resulted in a distinctive increase of Bacteroidetes, Actinobacteria, Verrucomicrobia, and Proteobacteria during the study (Figure 4.2). Although there were no or slight differences among groups for the three most abundant phyla, long-term melanoidin consumption resulted in a significant increase of Actinobacteria in the group that received 100% of melanoidin malts (Figure 4.3). Increased abundance of Verrucomicrobia was observed in groups fed 50%, 75%, and 100% of melanoidin malts at day 21 (Figure 4.3). Regardless of treatment, the abundance of *Dorea, Oscillibacter*, and *Alisitpes* were decreased, but the relative abundance of *Lactobacillus*, *Paresutterella*, *Akkermansia*, *Bifidobacterium*, and *Barnesilla* were increased during the study (Figure 4.4). In addition, there were no significant differences at day 0 among groups of all genera, except that the 25% group had significantly higher *Bacteroides* and *Parasutterella* and lower *Alistipes.*

Several genera among the Firmicutes were found to be significantly affected by the amount of melanoidins from day 3 to day 21. The relative abundance of *Clostridium XIVa* generally increased at days 3 and 7, with significantly lower abundances in high melanoidins groups, but the differences decreased over the long-term (Figure 4.5A). The abundance of *Dorea* was higher at day 0 but after melanoidin malts consumption, *Dorea* decreased and resulted in slight or non-significant differences among groups at days 3, 7, 14, and 21 (Figure 4.5B). *Clostridium XIVb, Roseburia,* and *Lactobacillus* resulted in gradual significant increases at days 3, 7, 14, and 21. Lower abundances of *Clostridium XIVb* and *Lactobacillus* were found in the high melanoidin groups compared to other groups at days 7 and 14 (Figure 4.5B & C). The 75% and 100% groups had a higher abundance of *Roseburia* at day 14 (Figure 4.5B). The relative abundance of *Oscillibacter* decreased at days 3, 7, 14, and 21, but there was no significant differences among groups (Figure 4.5D). Long-term melanoidin consumption resulted in a significant decrease of

*Pseudoflavonifractor* at day 21, and there were no significant differences among groups (Figure 4.5E). *Ruminococcus* was found in high abundance at days 14 and 21 in the 0% group, which was significantly different from the 100% group (Figure 4.5E). *ClostridiumIV* showed a slight difference among groups at days 3 and 7 (Figure 4.5E).

The melanoidin malt consumption resulted in significant increases in *Barnesiella* (Bacteroidetes) at days 7, 14, and 21. *Barnesiella* abundance was significantly higher in low percentage melanoidin malts (Figure 4.6). The relative abundance of *Alistipes* was significantly depleted during the study at days 3, 7, 14, and 21 although abundances were slightly different among groups (Figure 4.6). The relative abundance of *Bacteroides* resulted in an increase during the study and a sharp increase showed in the 25% group (Figure 4.6). The abundant genus of Actinobacteria was *Bifidobacterium,* which increased throughout the study, with significantly higher abundance for the 100% group that consumed melanoidin malts (Figure 4.7A). The consumption of melanoidin malts resulted in a gradual increase of *Akkermansia* at days 3, 7, 14, and 21. There was significant difference among groups at days 3 and 21, and the high abundance of *Akkermansia* was in the group that consumed 100% of melanoidin malts (Figure 4.7B). The responsive genus among Proteobacteria was *Parasutterella*; which exhibited a sharp significant increase throughout the study, but we observed only slight differences among groups (Figure 4.7C).

The relative abundance of several Firmicutes genera (*Clostridium XIVb* and *Lactobacillus*) (Figure 8 A&C), as well as *Bifidobacterium* (Figure 8E), and *Akkermansia* (Figure 8F) were increased significantly in all groups that consumed 0%, 25%, 50%, 75%, and 100% of melanoidin malts throughout the study from day 0 to day 21, and there were slight or significant difference among days ($P < 0.005$). Significant increases in *Parasutterella* were observed in the mice that

were fed 0%, 25%, and 50% of melanoidin malts during the study, while the relative abundance of *Parasutterella* was extremely low in groups that received 75% and 100% throughout the study (Figure 8G). The relative abundance of *Dorea* (Firmicutes; Figure 8B) and *Alistipes* (Bacteroidetes; Figure 8D) were decreased gradually through the study from 0 day to 21 day in all mice that were fed 0%, 25%, 50%, 75%, and 100% of melanoidin malts, and there were significant differences between days ($P < 0.005$).

**4.5 Discussion and Conclusion:**

The purpose of this study was to investigate the impact of melanoidins-rich malts, which may represent a major source of specific dietary melanoidins for humans, on the composition of the gut microbiota and their potential prebiotic effects. During food processing, melanoidins are the final products of the Maillard reaction and have been suggested to exert health benefits due to their purported antioxidant, antimicrobial, antihypertensive, and prebiotic properties [6,36]. Melanoidins isolated from coffee and biscuits have been shown to damage the outer membrane of a pathogenic *E.coli* strain [22]. In addition, roasted barley produced strong antioxidant properties [37]. It has been reported that a large proportion of the HMW melanoidins are excreted in feces and urine [14]. We noted the brownish color of the feces and urine from mice that fed 100% of melanoidin malts, compared to mice that consumed 0% of melanoidin malts after 21 days in the present study. Several studies reported that the dietary melanoidins could escape digestion and pass through the gastrointestinal tract where they may be fermented by the intestinal microbiota [14,17,38]. Dietary melanoidins have already been suggested as behaving like dietary fiber by enhancing the growth of beneficial gut bacteria [36].

The consumption of coffee melanoidins had no effect on the weight gain of rats that were being fed a high-fat diet [39]. However, the consumption of germinated barley (malt) resulted in

significant decreases in body weight in mice [40]. In this study, we found that the average daily gain was initially increased but subsequently lowered by the consumption of melanoidins-rich malts, confirming that dietary melanoidins have limited impact on weight gain.

The quantification of SCFAs in feces is a useful index of the fermentative potential of the gut microbiota. In this study, the proportion of acetate was stable during the study, but the quantity of propionic and butyrate was decreased. The concentration of butyrate, acetate, and propionate were higher in mice that consumed 100% of melanoidin malts, compared to 0% of melanoidin malts at day 14 and 21, respectively. The total SCFAs were stable in mice that received 100% of melanoidin malts (4.68 mmol/L), compared to mice that consumed 0% of melanoidin malts (2.29 mmol/L) at 21 days with a significant decrease. Along the same line as, the total production of SCFAs was increased in rats that were fed bread crust (81.0 μmol/g), compared to controls (42.7μmol/g) [41].There was a significant increase of propionate in rats that received bread crust, but butyrate and acetate were decreased [41]. These results are in favor of the hypothesis that melanoidins modulate the gut microbiome and fermentation patterns in a similar fashion than dietary fibers.

In this study, the relative abundance of Firmicutes decreased, but Bacteroidetes, Verrucomicrobia, Acinobacteria, and Proteobacteria increased during the study, which is consistent with a previous study that included higher abundance of Verrucomicrobia and Acinobacteria and lower abundance of Firmicutes in rats fed barley malt [23]. The impact of two barley products, whole-grain barley and barely malt with different chemical structures, on cecal microbiota in rats fed a high-fat diet has been reported [23]. Zhong *et al* found that consumption of whole-grain barley resulted in the increase of *Akkermansia* spp. and *Ruminococcus* spp. while *Roseburia* spp. and *Lactobacillus* spp. were more abundant in rats fed barley malt, compared to the control group that was enriched in *Oscillospira* spp. and *Dorea* spp. [23]. We observed that

*Ruminococcus* spp. and *Lactobacillus* spp. had a higher abundance in mice fed 0% of melanoidin malts. However, there was a significant decrease of *Dorea* and *Oscillibacter* throughout the study, which may be in line with a previous study of the reduction of some genera belonging to Firmicutes, such as *Dorea* after oral supplementation of glutamine [42]. A significant increase of *Roseburia* spp. was observed during the study, which may be in line with a study on *Roseburia* growth in healthy humans who consumed whole-grain barley for 60 days [43]. The effects of dietary fiber sources, alfalfa diet, showed an increase of *Clostridium* cluster XIVb, compared to the pure cellulose diet of suckling piglets [44]. We indeed observed increases of *Clostridium* cluster XIVb during the study with slight differences among groups.

In this present study, we observed a significant increase of *Bacteroides* spp. in mice that were fed fewer melanoidin malts, especially 25% of melanoidin malts which may be in line with the previous *in vitro* report of an increase in the proportion of *Bacteroides* spp. in light and medium roasted coffee, compared to dark roasted coffee [28]. *Bacteroides* spp. are known for their ability to ferment different mucin polysaccharides because they possess a wide range of carbohydrate-depolymerizing enzymes [45]. Moreover, in the present study, we observed the significant difference of average daily gain and proportion consumed found in mice that consumed 25%, which had a higher abundance of *Bacteroides* spp. The relative abundance of genus *Barnesiella* spp. were found at low levels, which makes up less than one percent of an individual's total gut bacteria; they, in particular, are known for their ability to control the spread of highly antibiotic-resistant bacteria [46,47]. Significant decreases of *Barnesiella* were detected in a guinea pig model fed Western diets associated with metabolic syndrome [48]. However, dietary resistant starch resulted in significant increases of *Barnesiella*, *Ruminococcus*, and *Bifidobacterium* in a rodent colitis-associated colorectal cancer model, which suggested resistant starch might have a beneficial effect

on patients with ulcerative colitis [49]. In the present study, significant increases of *Barnesiella* were observed, especially in mice fed 0% and 25% at 21 days. Decreased abundance of *Alistipes* showed in the present study. Wang et al similarly showed that oligosaccharide treatment decreased the levels of *Alistipes* of mice with constipation [50].

*Akkermansia* spp. are known as mucin-degrading bacteria that use glycated proteins as an energy source [51]. *Akkermansia muciniphila* is known for their ability of anti-inflammatory effects in the intestine. A significant decrease of *A. muciniphila* was found in colitic mice [52]. We detected a high abundance of *Akkermansia* spp in mice that were fed melanoidin-rich malts. *Bifidobacterium* spp. were also found in high abundance in the melanoidin-rich-malt group. Dietary fiber can enhance the growth of *Bifidobacterium* spp.[36]. Coffee consists of soluble fiber, mainly galactomannans and arabinogalactans [28]. The roasted coffee silverskin, which has 60% total dietary fiber, enhanced preferential growth of *Bifidobacterium* spp. *in vitro* compared to other anaerobic bacteria [53]. An increase in the population of *Bifidobacterium* spp. was also shown after the consumption of coffee in humans [54]. In addition to coffee, bread crust melanoidins promoted the growth of *Bifidobacterium* spp. using a static batch culture [26]. The type of melanoidins plays an important role in enhancing the growth of *Bifidobacterium* spp. Coffee melanoidins are characterized by a considerable carbohydrate, but bread crust melanoidins consist of the prevalence of amino acids [26]. Thus, the coffee melanoidins increased the growth of *Bifidobacterium* spp., compared to bread crust melanoidins [26]. The structures of melanoidin malts are similar to coffee melanoidins by containing a considerable amount of carbohydrates and fibers. Furthermore, distinct increases of *Parasutterella,* known as saccharolytic strain, were detected during study, which might be in line with previous reports of the proportion of *Parasutterella* that were elevated by carbohydrate solutions consumption on rodent models [55].

We conclude that the long term consumption of melanoidin malts increased beneficial microorganisms, such as *Bifidobacterium*, *Akkermansia*, and *Lactobacillus* although there were no significant differences in the population of *Lactobacillus* between groups that consumed 0% and 100% of melanoidin malts. These results confirm that gut microbiota responds differently to different melanoidins-rich food, and that melanoidins-rich malts appear to exert particularly beneficial changes, a property that could potentially lead to the development of novel prebiotic foods.

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**Table 4.1:** Temporal variation of Feed intake for all mice considered as one group. Data are expressed as mean ± SEM. *P* value <0.05 was considered to be significant difference (indicated by superscript letters):

| **Feed intake by days (g/day).** | **Estimated Mean** |
|---|---|
| 4 days | $0.53\pm0.014^{b}$ |
| 7 days | $0.74\pm0.012^{a}$ |
| 11 days | $0.46\pm0.014^{cd}$ |
| 14 days | $0.45\pm0.014^{d}$ |
| 18 days | $0.49\pm0.014^{c}$ |
| 21 days | $0.33\pm0.013^{f}$ |
| 25 days | $0.38\pm0.013^{e}$ |

**Table 4.2:** Effect of diet on average daily gain. Data are expressed as mean ± SEM:

| **Average Daily Gain (g/day)** | **7 days** | **18 days** | **25 days** |
|---|---|---|---|
| Free-Melanoidins | $0.17\pm0.068^{abcA}$ | $0.19\pm0.068^{aA}$ | $0.31\pm0.068^{abA}$ |
| 25% Melanoidins | $0.048\pm0.068^{cB}$ | $0.34\pm0.068^{aA}$ | $0.47\pm0.068^{aA}$ |
| 50% Melanoidins | $0.28\pm0.068^{abA}$ | $0.18\pm0.068^{aA}$ | $0.18\pm0.068^{bA}$ |
| 75% Melanoidins | $0.34\pm0.068^{aA}$ | $0.20\pm0.068^{aAB}$ | $0.14\pm0.068^{bB}$ |
| 100% Melanoidins | $0.13\pm0.068^{bcA}$ | $0.19\pm0.068^{aA}$ | $0.23\pm0.068^{bA}$ |

Different letters indicate significant difference ($P<0.05$; ANOVA)

The lowercase letters indicate significant difference ($P<0.005$; ANOVA) in the same day with different groups

The capital letters indicate significant difference ($P<0.05$; ANOVA) in the same group with different days

**Table 4.3:** Effects of melanoidin malts on short-chain fatty acids. Data are expressed as mean ± SEM:

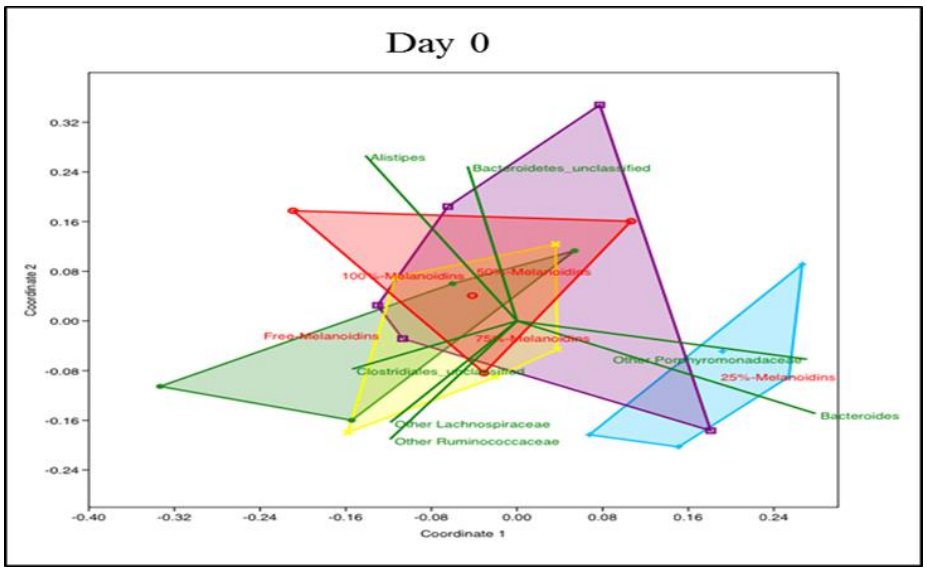
| | **Melanoidins** | **day 0** | **day 3** | **day 7** | **day 14** | **day 21** |
|---|---|---|---|---|---|---|
| Acetate | 0% | 4.01±0.68$^{a}$ | 3.1±0.94$^{aA}$ | 4.1±1.33$^{aA}$ | 2.35±0.97$^{aA}$ | 2.13±0.63$^{aB}$ |
| (mmol/L) | 100% | 4.01±0.68$^{a}$ | 3.7±1.33$^{aA}$ | 3.6±1.41$^{aA}$ | 4.14±1.05$^{aA}$ | 4.51±0.43 $^{aA}$ |
| Propionate | 0% | 0.25±0.03$^{a}$ | 0.21±0.03$^{aA}$ | 0.16±0.04$^{aA}$ | 0.07±0.03$^{bA}$ | 0.06±0.01$^{bB}$ |
| (mmol/L) | 100% | 0.25±0.03$^{a}$ | 0.14±0.05$^{aA}$ | 0.11±0.04$^{bA}$ | 0.09±0.02$^{bA}$ | 0.09±0.03 $^{bA}$ |
| Butyrate | 0% | 0.42±0.17$^{a}$ | 0.27±0.04$^{aA}$ | 0.18±0.07$^{aA}$ | 0.06±0.03$^{bB}$ | 0.10±0.01$^{bA}$ |
| (mmol/L) | 100% | 0.42±0.17$^{a}$ | 0.13±0.04$^{bA}$ | 0.09±0.02$^{bA}$ | 0.14±0.01$^{bA}$ | 0.08±0.02 $^{bA}$ |
| Total SCFAs | 0% | 4.7±0.88 | 3.6±1.01 | 4.44±1.44 | 2.5±1.03 | 2.29±0.62 |
| | 100% | 4.7±0.88 | 3.8±1.42 | 3.8±1.5 | 4.4±1.08 | 4.68±0.48 |

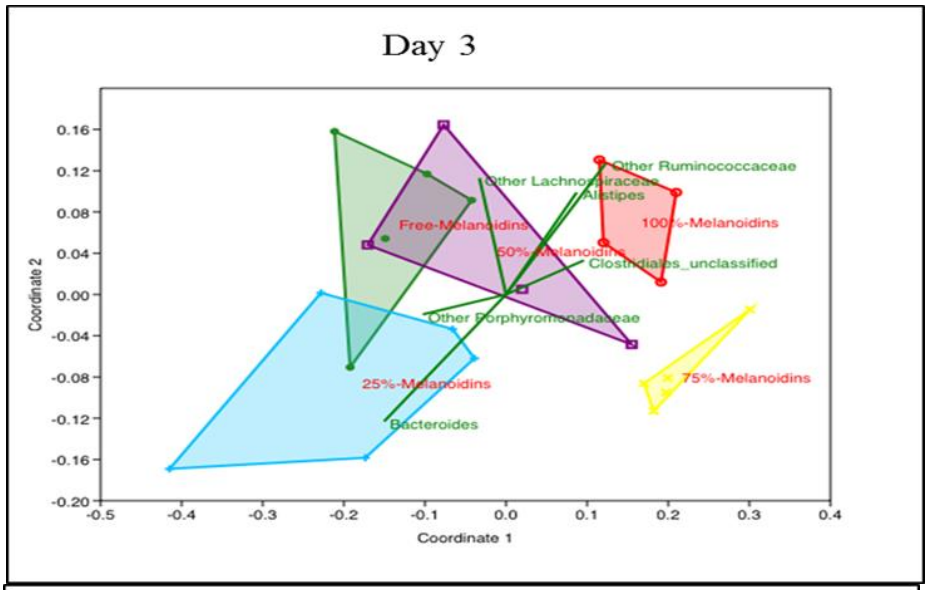
Same letters indicate no significantly difference

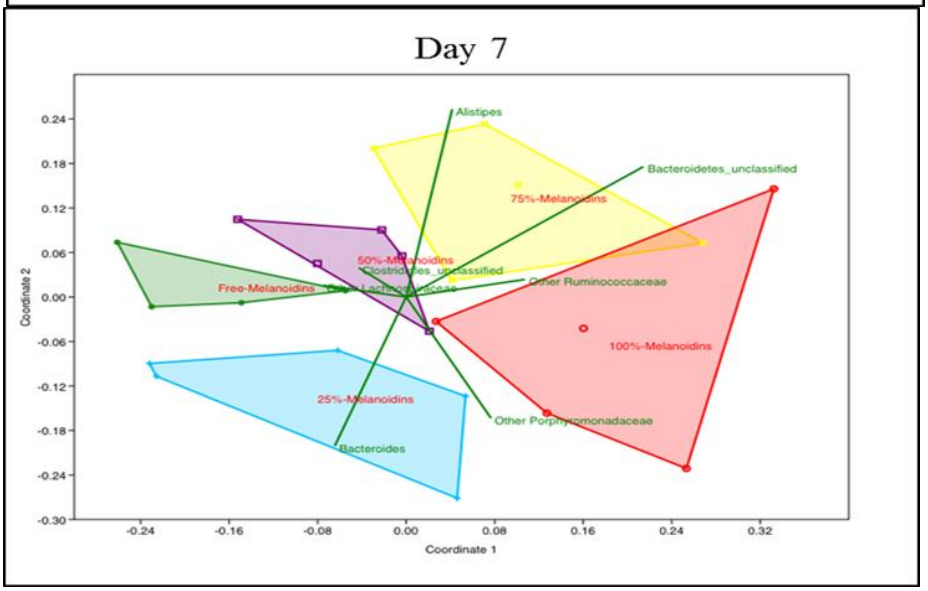
Different letters indicate significantly difference ($P$<0.05; ANOVA)

The small letters indicate significantly difference ($P$<0.005; ANOVA) in the same diet with different days

The capital letters indicate significantly difference ($P$<0.05; ANOVA) in the same day with different diets


Day 0
Alistipes
Bacteroidetes_unclassified
100%-Melanoidins
50%-Melanoidins
Free-Melanoidins
75%-Melanoidins
Other Porphyromonadaceae
Clostridiales_unclassified
25%-Melanoidins
Bacteroides
Other Lachnospiraceae
Other Ruminococcaceae
Coordinate 2
Coordinate 1


Day 3
Other Ruminococcaceae
Other Lachnospiraceae
Alistipes
Free-Melanoidins
100%-Melanoidins
50%-Melanoidins
Clostridiales_unclassified
Other Porphyromonadaceae
75%-Melanoidins
25%-Melanoidins
Bacteroides
Coordinate 2
Coordinate 1


Day 7
Alistipes
Bacteroidetes_unclassified
75%-Melanoidins
50%-Melanoidins
Clostridiales_unclassified
Free-Melanoidins
Other Ruminococcaceae
100%-Melanoidins
25%-Melanoidins
Other Porphyromonadaceae
Bacteroides
Coordinate 2
Coordinate 1

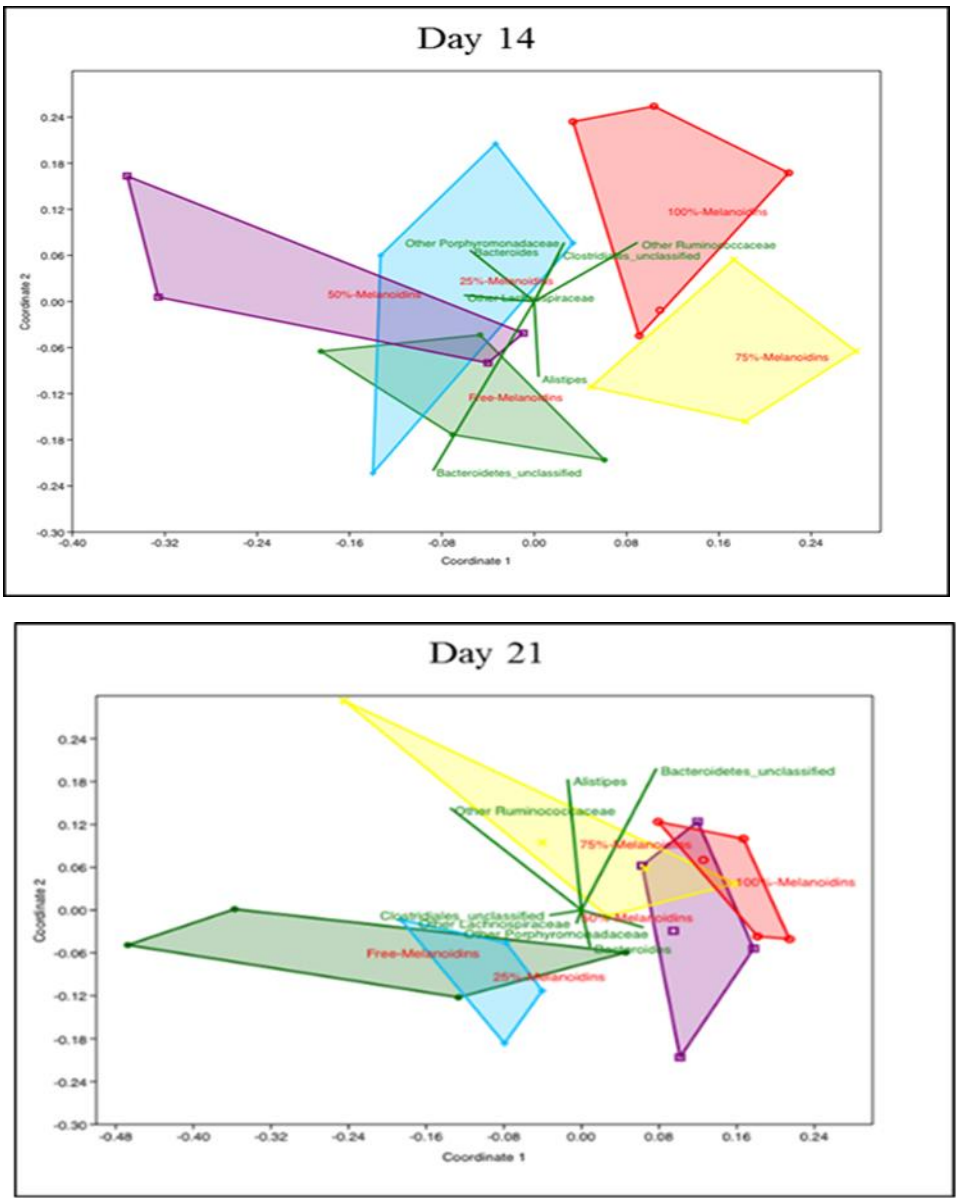
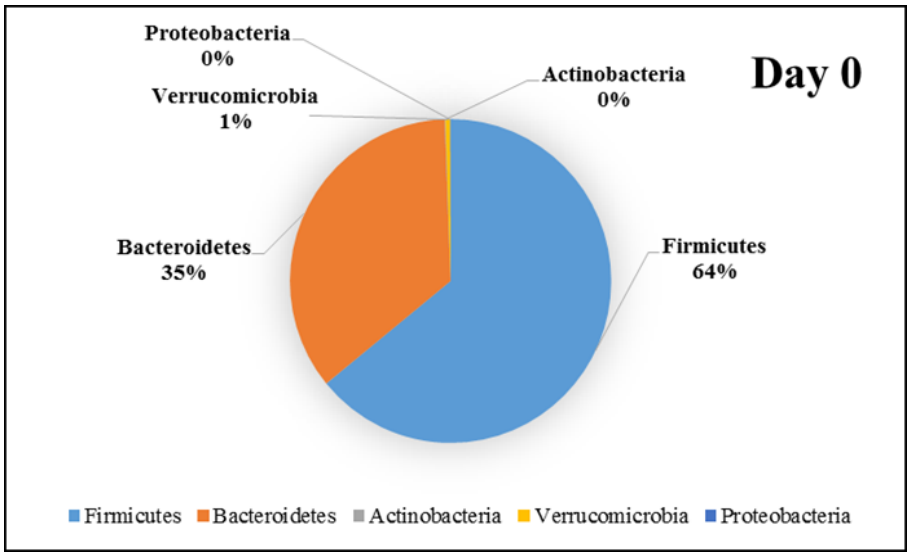
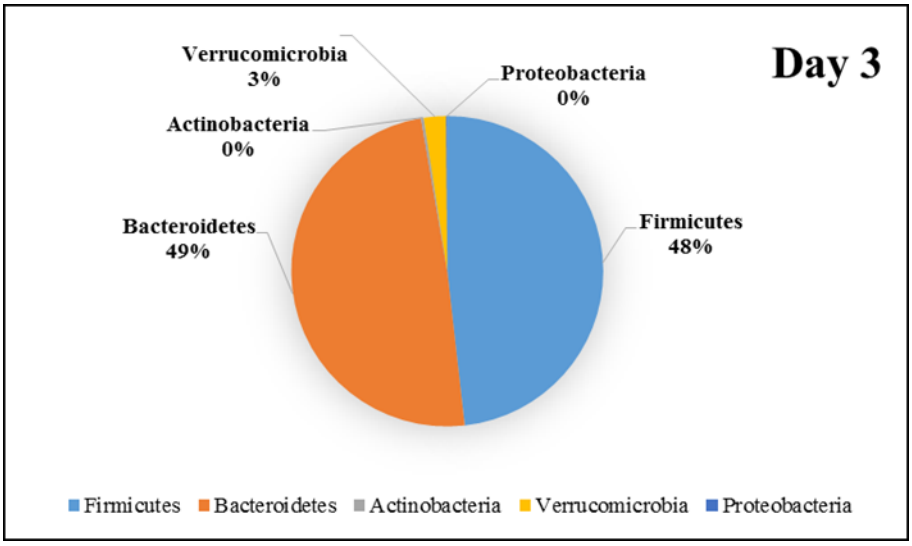
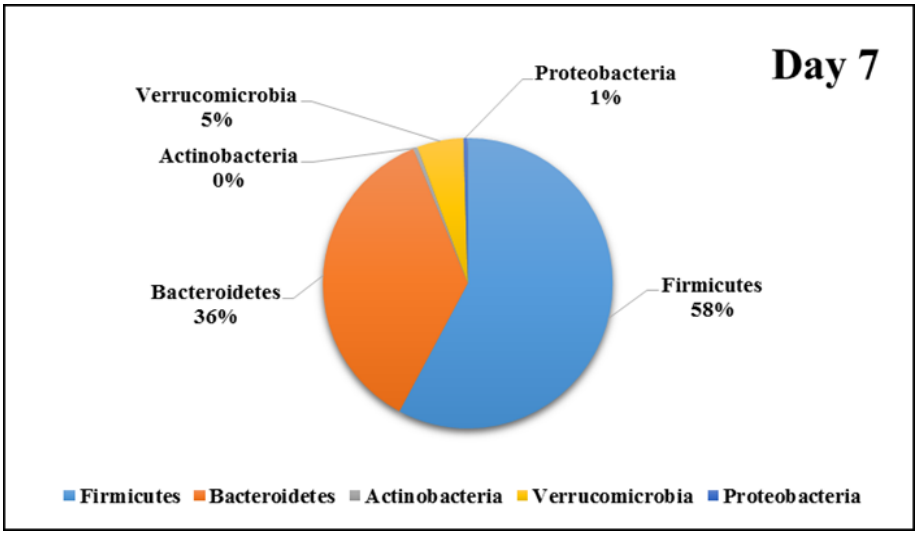
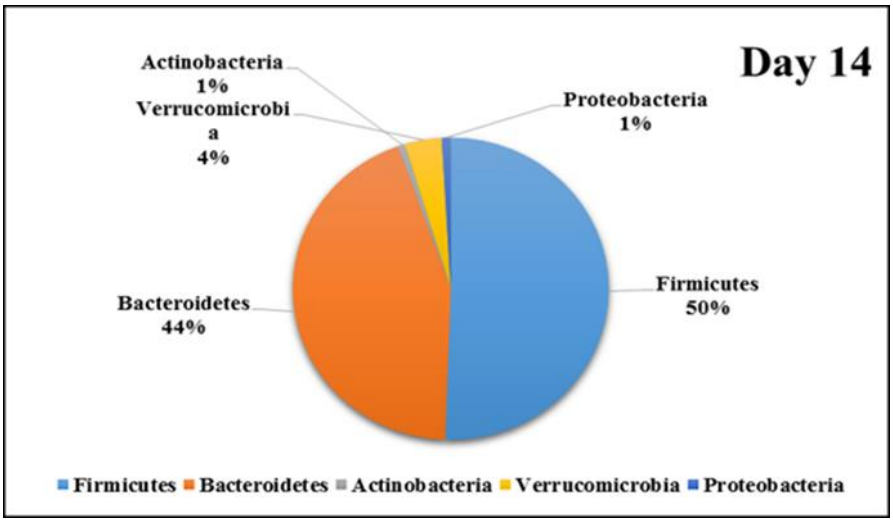



**Figure 4.1:** Impact of increasing dietary melanoidin malts on the composition of the gut microbiota (NMDS): Day 0 no significant difference (ANOSIM $P>0.05$); Day 3, 100% and 75% significantly different from 0% and 25% (ANOSIM $P<0.05$); Day 7, 25%, 50%, 75%, and 100% of melanoidins significantly different from 0% (ANOSIM $P<0.05$); Day 14, 0%, 25%, and 50% significantly different 75% and 100%; Day 21, 0% and 25% significantly different from 50%, 75%, and 100%.


Day 0
Proteobacteria
0%
Actinobacteria
0%
Verrucomicrobia
1%
Bacteroidetes
35%
Firmicutes
64%
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Proteobacteria


Day 3
Verrucomicrobia
3%
Proteobacteria
0%
Actinobacteria
0%
Bacteroidetes
49%
Firmicutes
48%
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Proteobacteria


Day 7
Proteobacteria
1%
Verrucomicrobia
5%
Actinobacteria
0%
Bacteroidetes
36%
Firmicutes
58%
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Proteobacteria



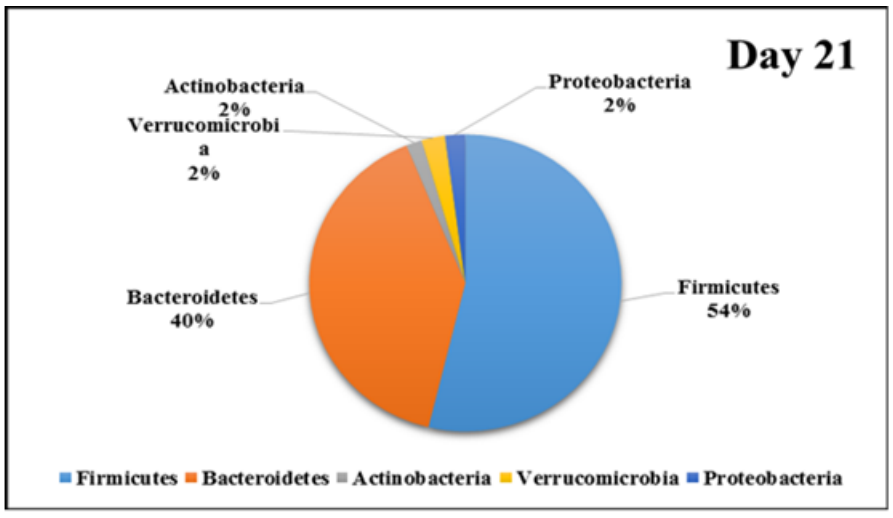
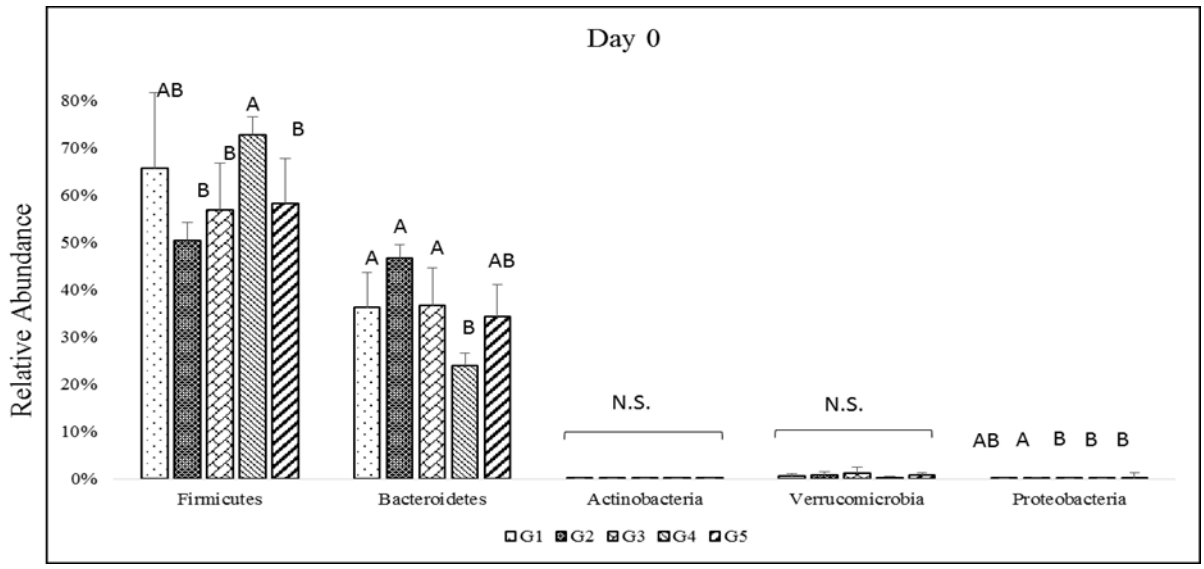
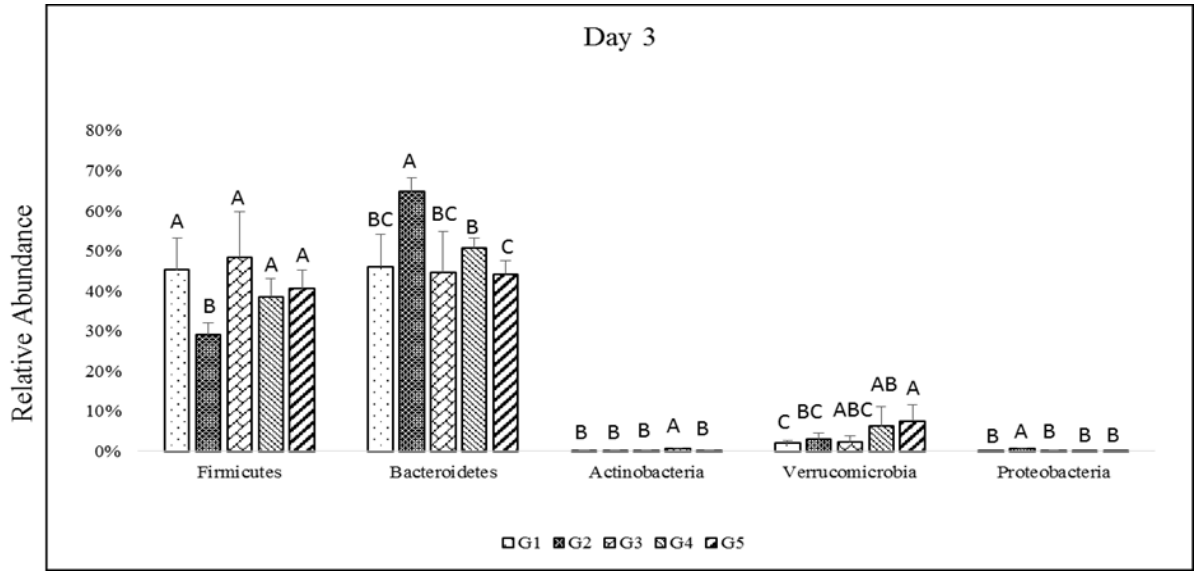
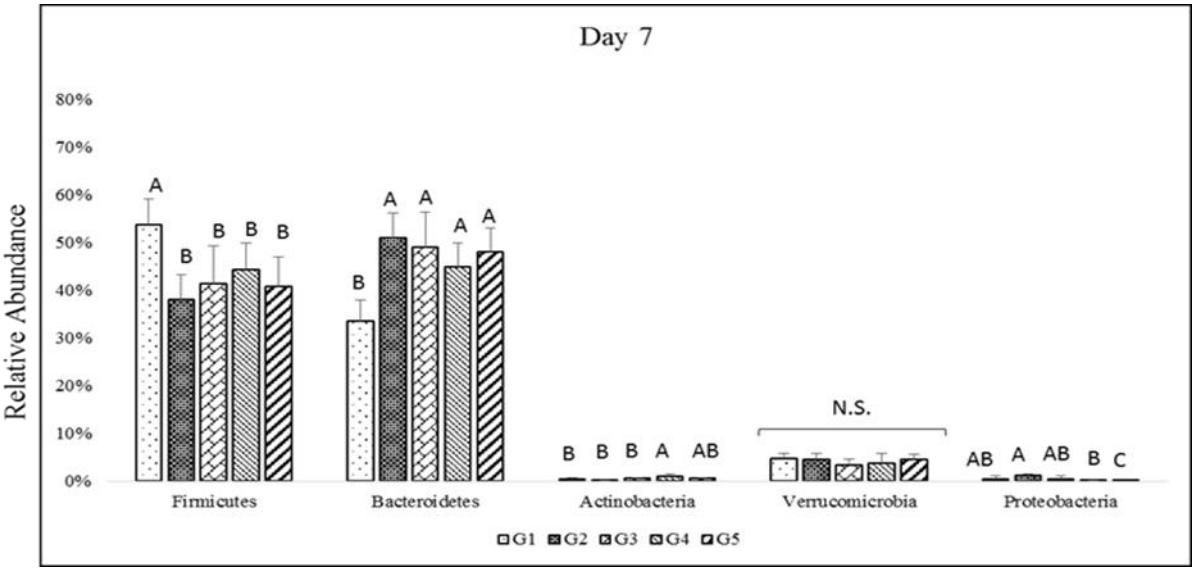




**Figure 4.2:** Impact of the consumption of malts (regardless of melanoidins content) on the abundant phyla during the study.


Day 0
Relative Abundance
80%
70%
60%
50%
40%
30%
20%
10%
0%
AB
B
B
A
B
A
A
A
B
AB
N.S.
N.S.
AB A B B B
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Proteobacteria
G1 G2 G3 G4 G5


Day 3
Relative Abundance
80%
70%
60%
50%
40%
30%
20%
10%
0%
A
B
A
A
A
BC
A
BC
B
C
B B B A B
C BC ABC AB A
B A B B B
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Proteobacteria
G1 G2 G3 G4 G5


Day 7
Relative Abundance
80%
70%
60%
50%
40%
30%
20%
10%
0%
A
B
B
B
B
B
A
A
A
A
B B B A AB
N.S.
AB A AB B C
Firmicutes
Bacteroidetes
Actinobacteria
Verrucomicrobia
Proteobacteria
G1 G2 G3 G4 G5

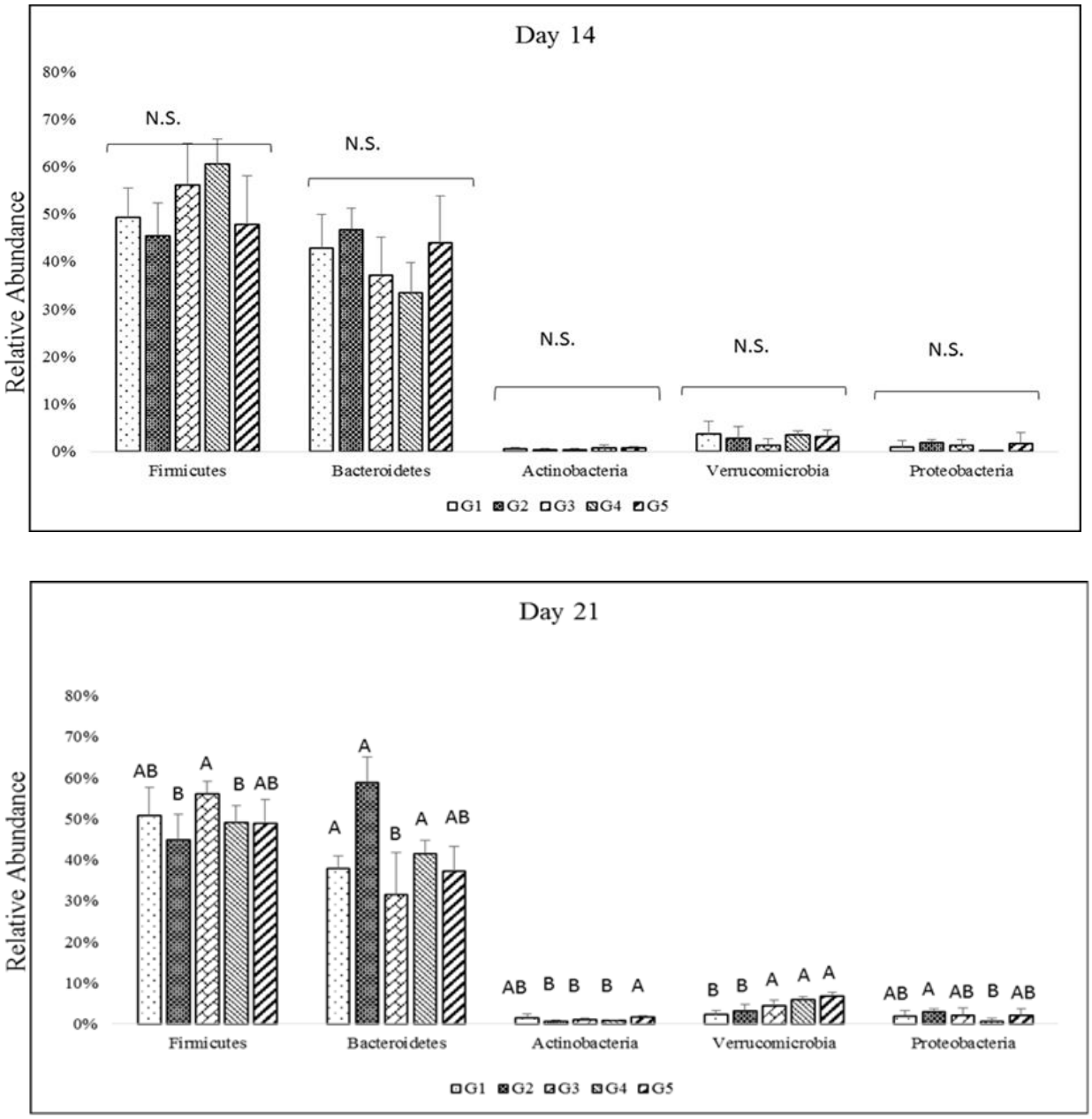
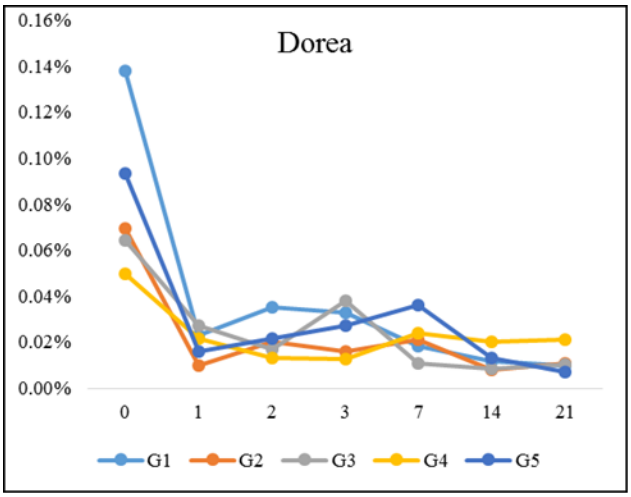
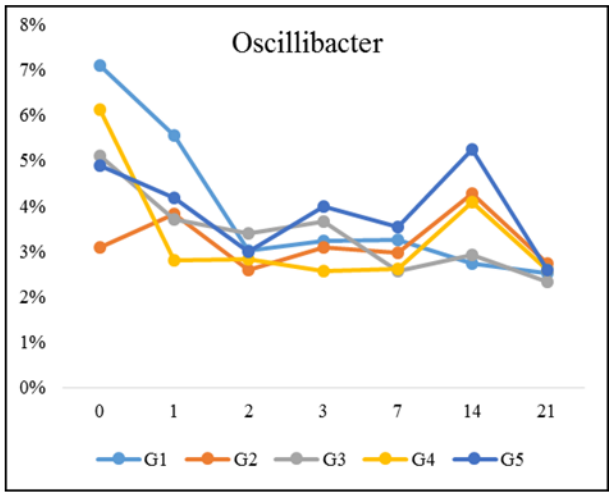
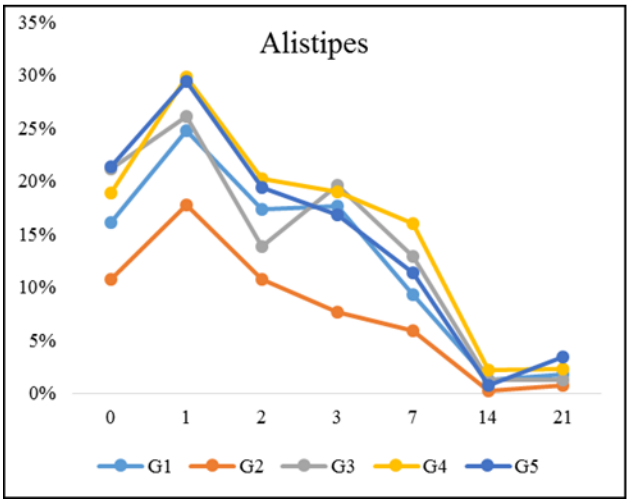
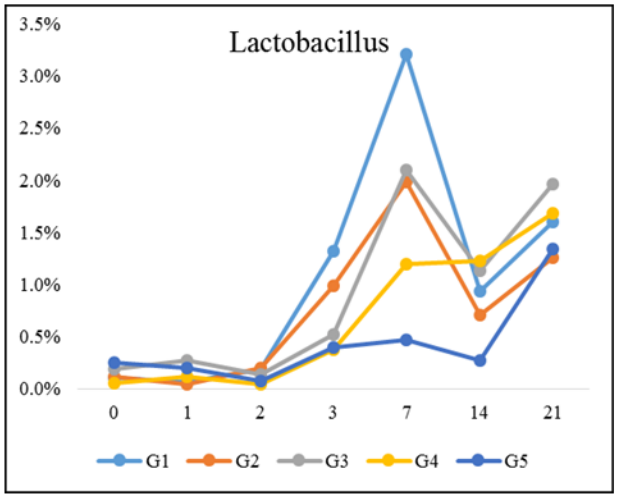



**Figure 4.3:** Impact of increasing dietary melanoidin malts of the composition of the gut microbiota at phylum level during the study. Significant differences ($P$<0.005) are indicated by different letters.


Dorea
0.16%
0.14%
0.12%
0.10%
0.08%
0.06%
0.04%
0.02%
0.00%
0
1
2
3
7
14
21
G1
G2
G3
G4
G5


Oscillibacter
8%
7%
6%
5%
4%
3%
2%
1%
0%
0
1
2
3
7
14
21
G1
G2
G3
G4
G5


Alistipes
35%
30%
25%
20%
15%
10%
5%
0%
0
1
2
3
7
14
21
G1
G2
G3
G4
G5


Lactobacillus
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
0
1
2
3
7
14
21
G1
G2
G3
G4
G5

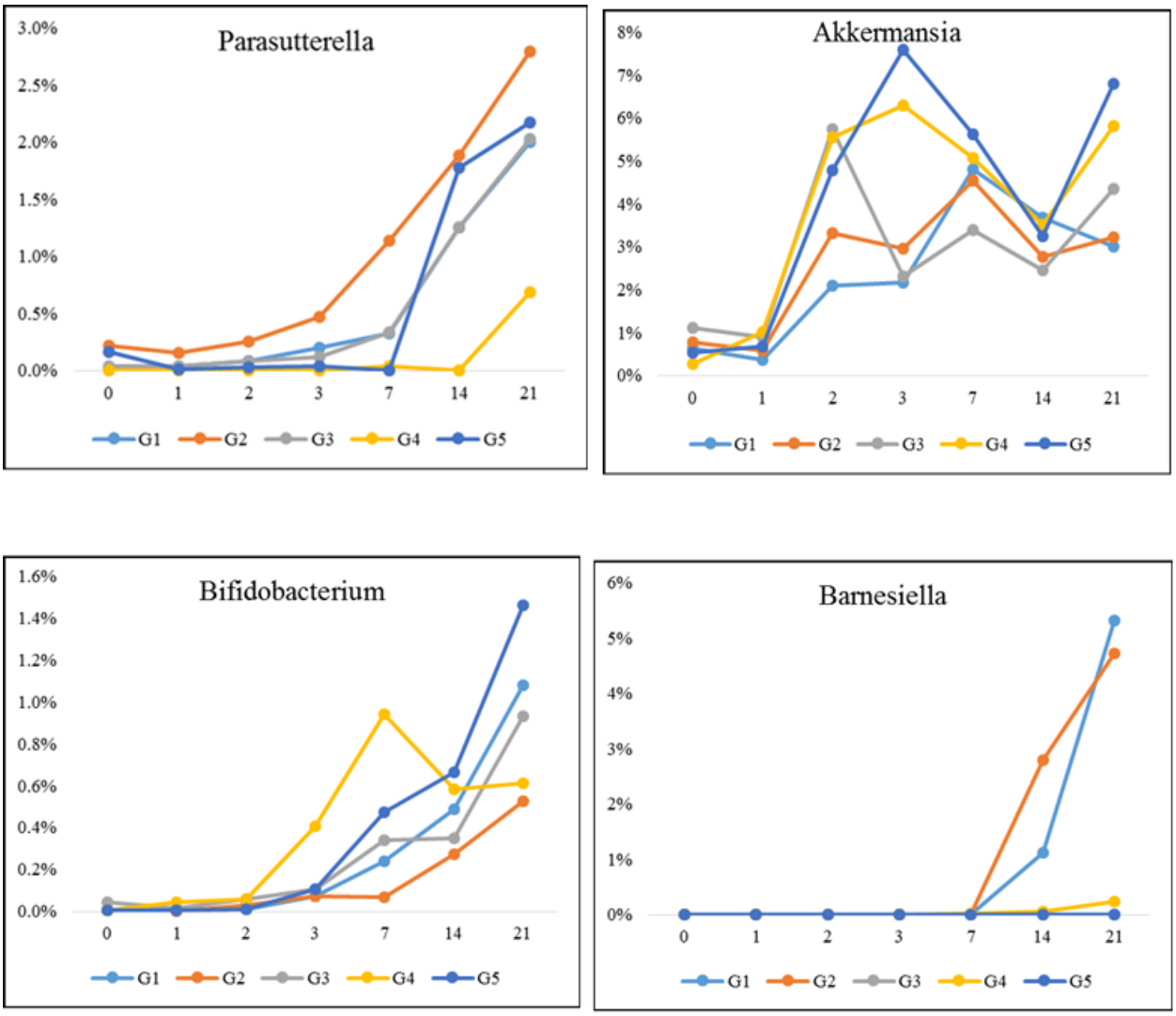
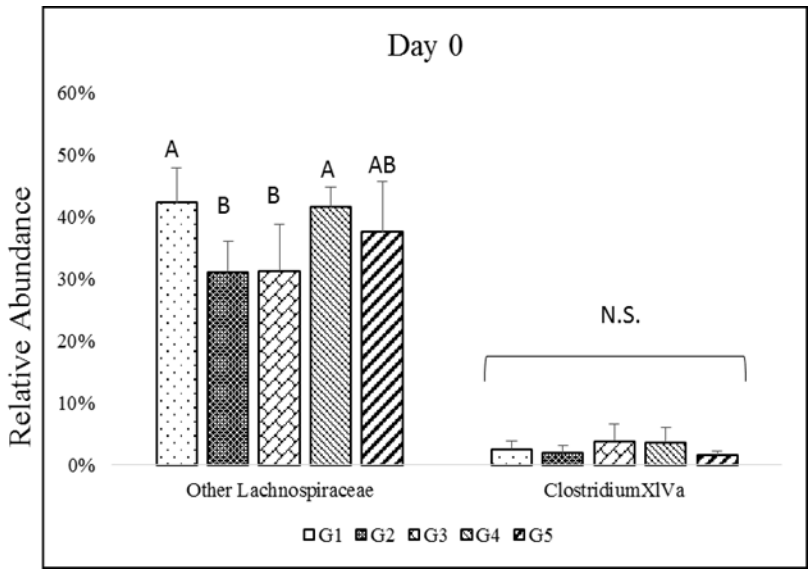
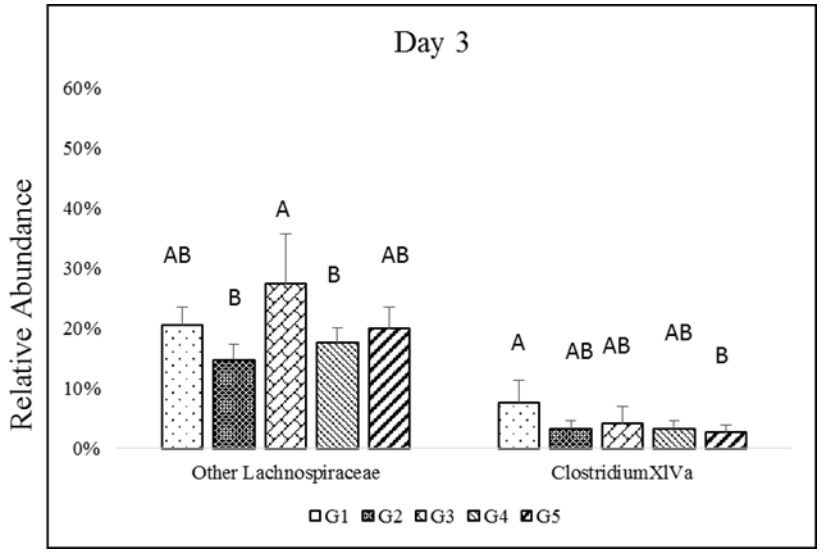
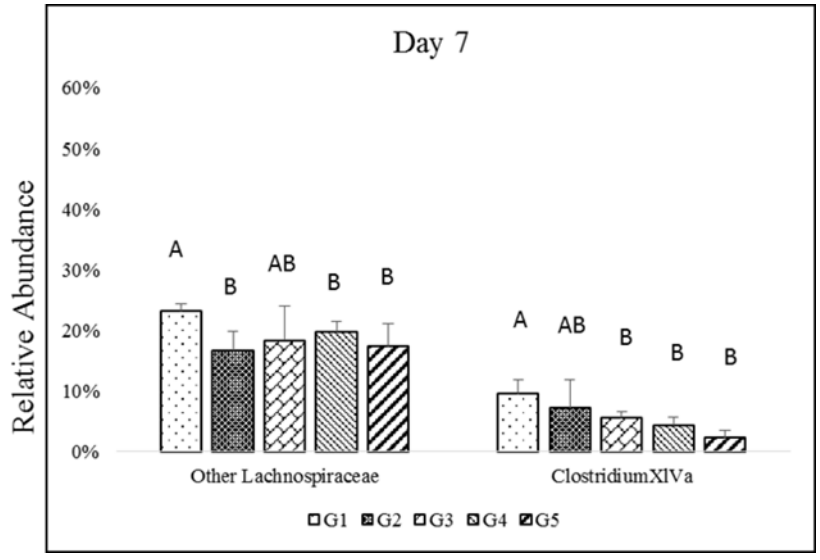



**Figure 4.4:** Impact of the consumption of melanoidin malts (regardless of melanoidins content) on the abundant genera during the study.


Day 0
Relative Abundance
60%
50%
40%
30%
20%
10%
0%
A
B
B
A
AB
N.S.
Other Lachnospiraceae
ClostridiumXlVa
G1 G2 G3 G4 G5


Day 3
Relative Abundance
60%
50%
40%
30%
20%
10%
0%
AB
B
A
B
AB
A
AB
AB
AB
B
Other Lachnospiraceae
ClostridiumXlVa
G1 G2 G3 G4 G5


Day 7
Relative Abundance
60%
50%
40%
30%
20%
10%
0%
A
B
AB
B
B
A
AB
B
B
B
Other Lachnospiraceae
ClostridiumXlVa
G1 G2 G3 G4 G5

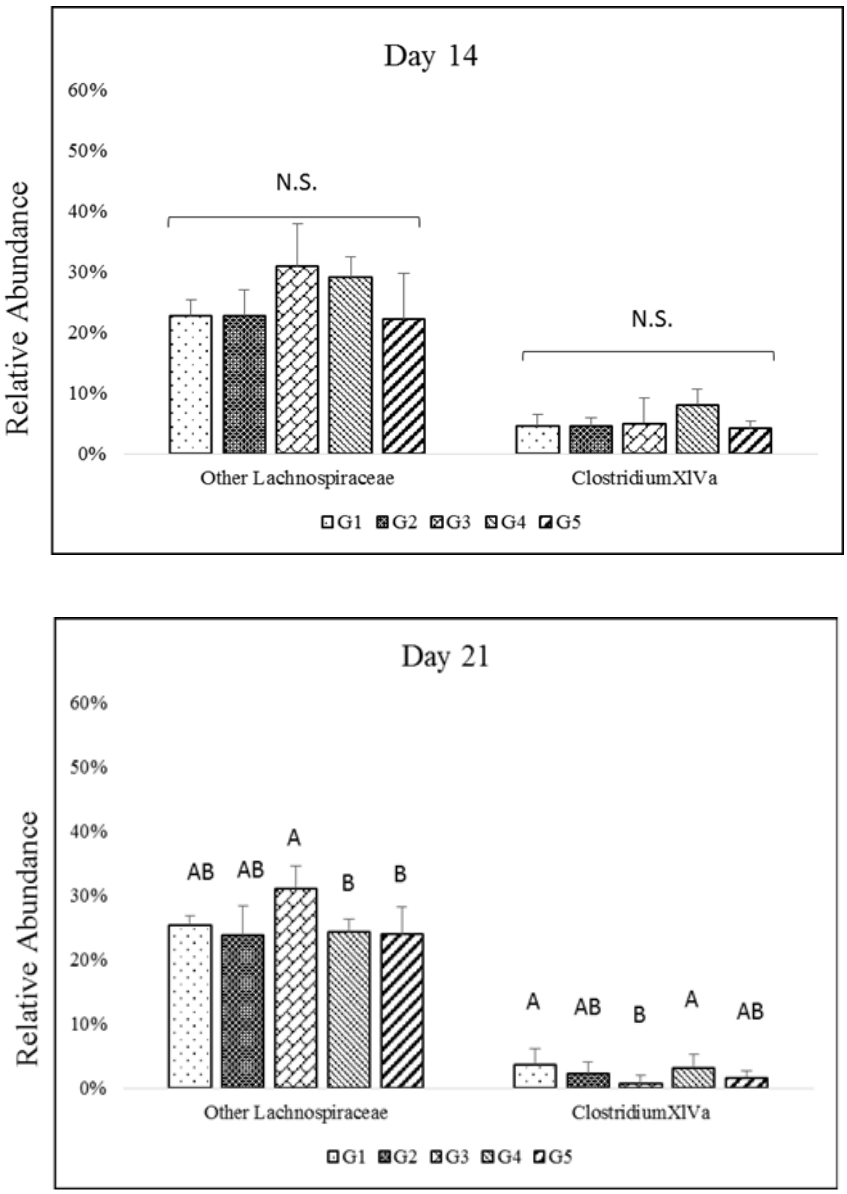
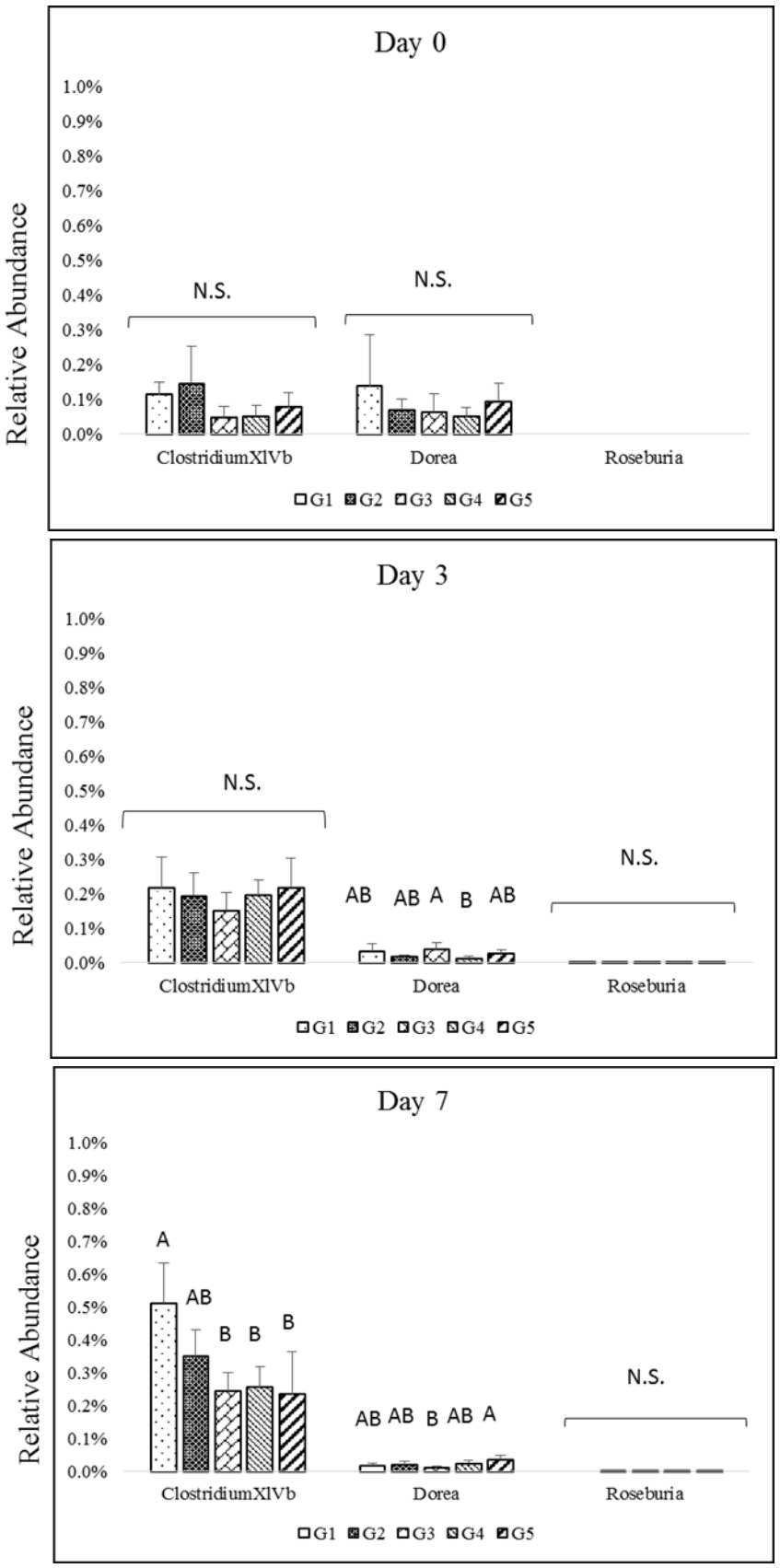



**Figure 4.5 A:** Impact of melanoidin malts on responsive genera relative abundance among the Firmicutes (Lachnospiraceae). Significant differences ($P$<0.005) are indicated by different letters.


Day 0
Relative Abundance
1.0%
0.9%
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
N.S.
N.S.
ClostridiumXlVb
Dorea
Roseburia
G1 G2 G3 G4 G5
Day 3
Relative Abundance
N.S.
AB AB A B AB
N.S.
ClostridiumXlVb
Dorea
Roseburia
G1 G2 G3 G4 G5
Day 7
Relative Abundance
A
AB
B B B
AB AB B AB A
N.S.
ClostridiumXlVb
Dorea
Roseburia
G1 G2 G3 G4 G5

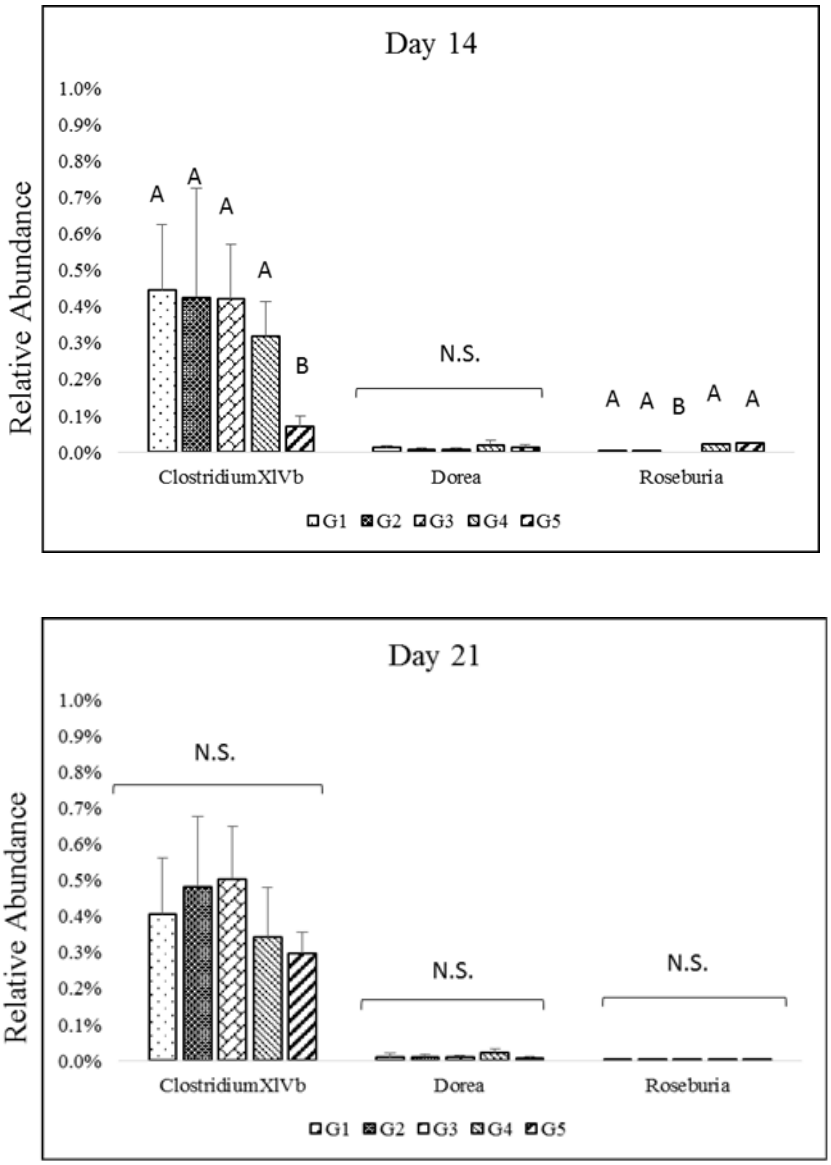
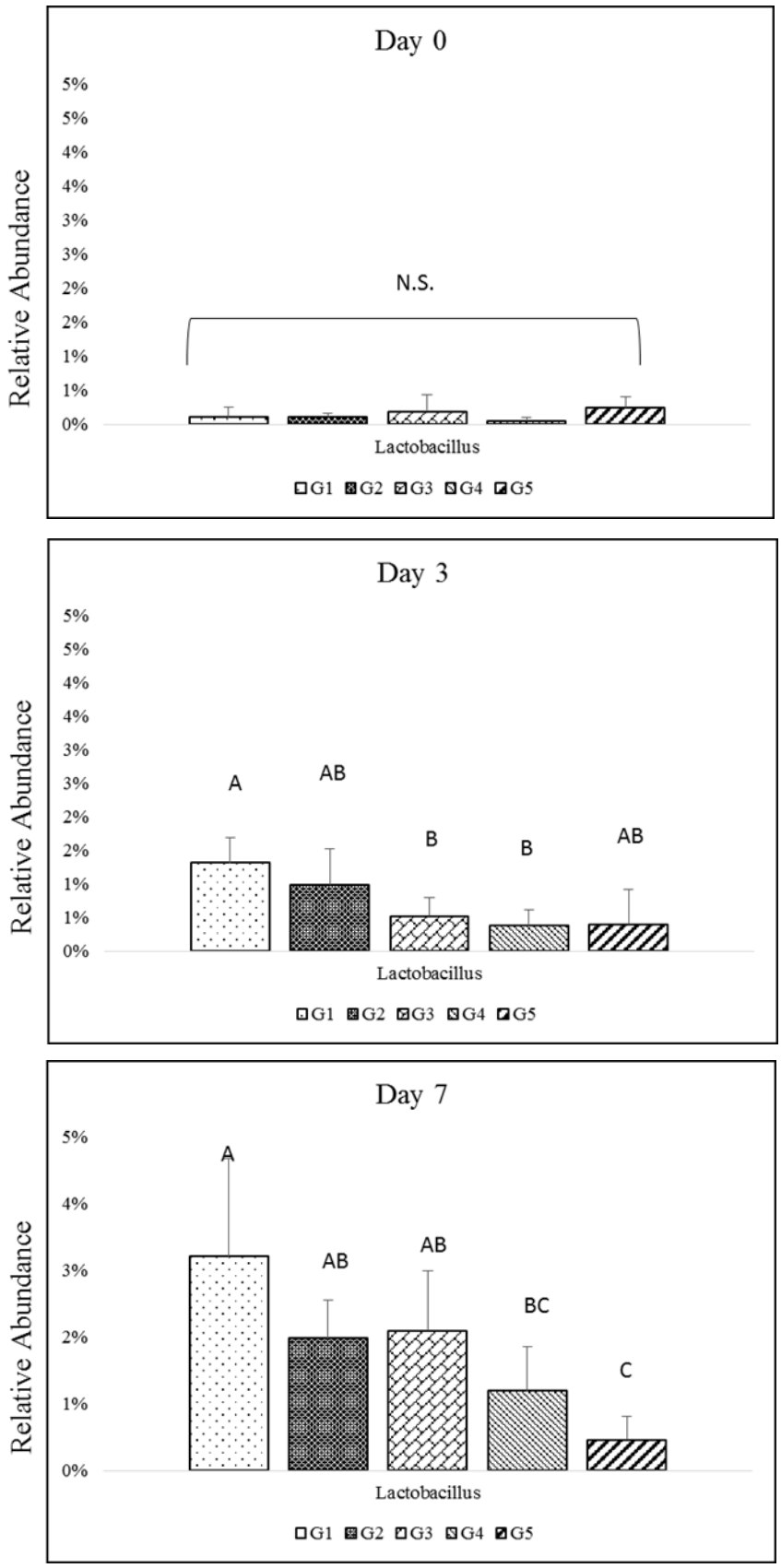



**Figure 4.5 B:** Impact of melanoidin malts on responsive genera relative abundance among the Firmicutes (Lachnospiraceae). Significant differences ($P<0.005$) are indicated by different letters.


Day 0
Relative Abundance
5%
5%
4%
4%
3%
3%
2%
2%
1%
1%
0%
N.S.
Lactobacillus
G1 G2 G3 G4 G5
Day 3
Relative Abundance
A
AB
B
B
AB
Lactobacillus
G1 G2 G3 G4 G5
Day 7
Relative Abundance
A
AB
AB
BC
C
Lactobacillus
G1 G2 G3 G4 G5

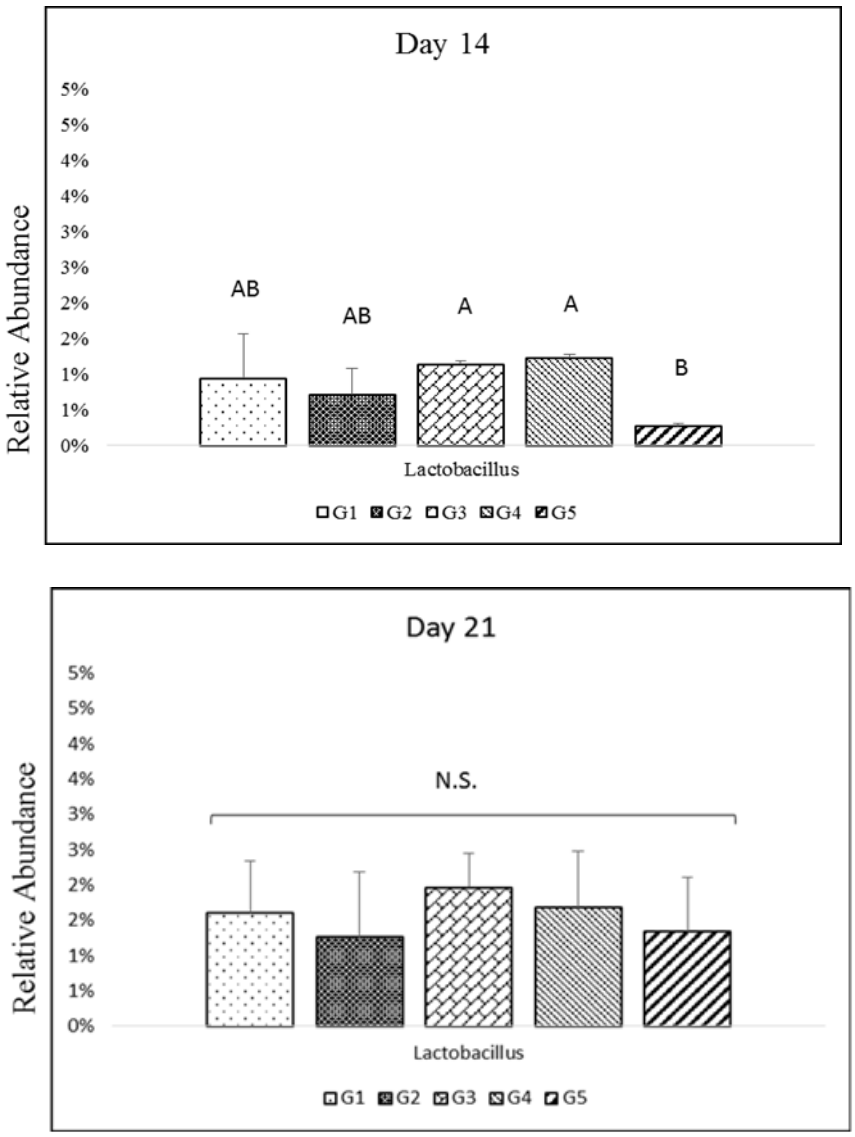
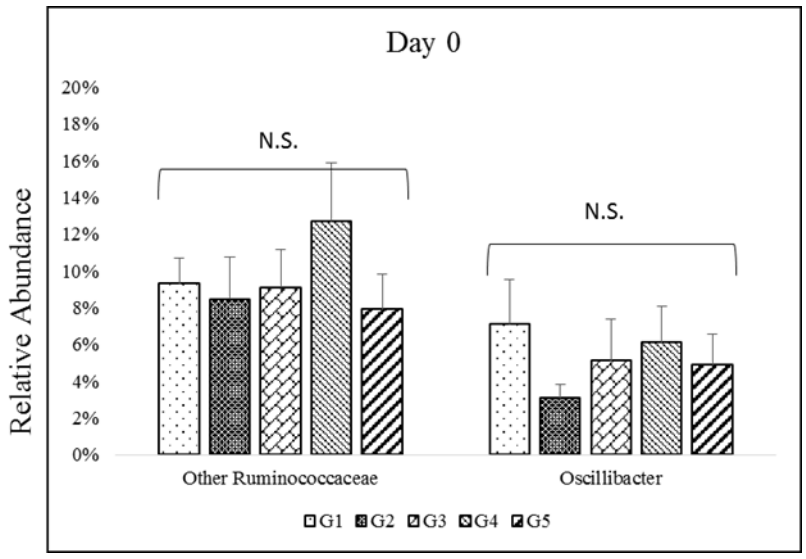
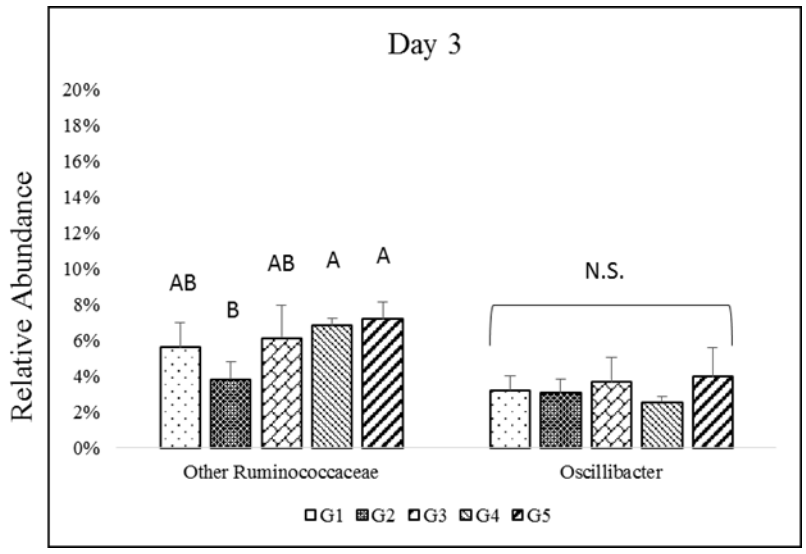
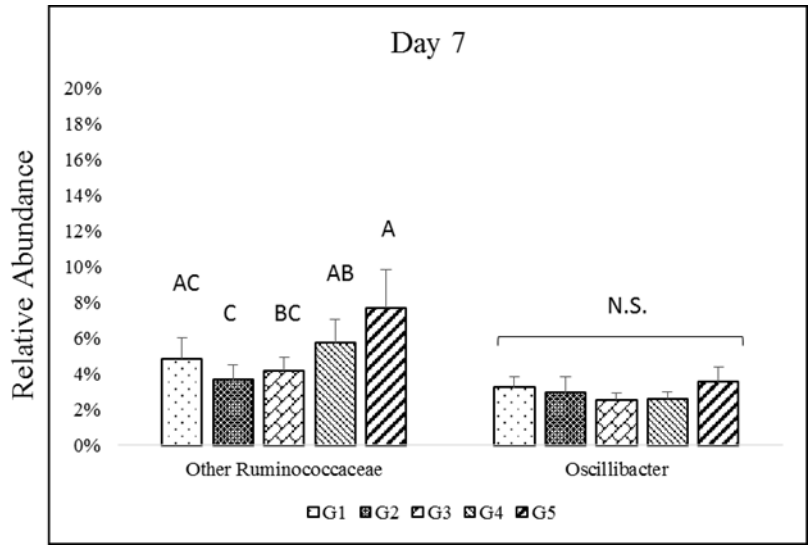



**Figure 4.5C**: Impact of melanoidin malts on responsive genera relative abundance among the Firmicutes (Lactobacillaceae). Significant differences ($P$<0.005) are indicated by different letters.


Day 0
Relative Abundance
20%
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
N.S.
N.S.
Other Ruminococcaceae
Oscillibacter
G1 G2 G3 G4 G5


Day 3
Relative Abundance
20%
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
AB
B
AB
A
A
N.S.
Other Ruminococcaceae
Oscillibacter
G1 G2 G3 G4 G5


Day 7
Relative Abundance
20%
18%
16%
14%
12%
10%
8%
6%
4%
2%
0%
AC
C
BC
AB
A
N.S.
Other Ruminococcaceae
Oscillibacter
G1 G2 G3 G4 G5

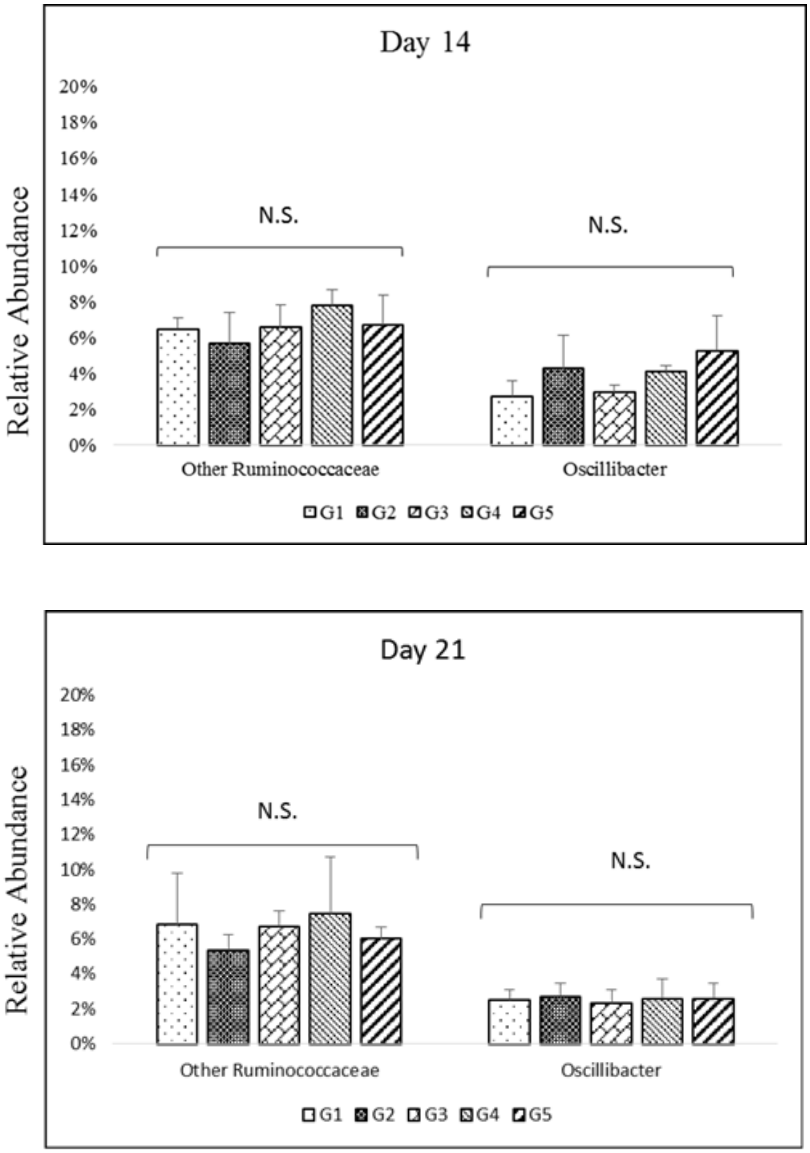
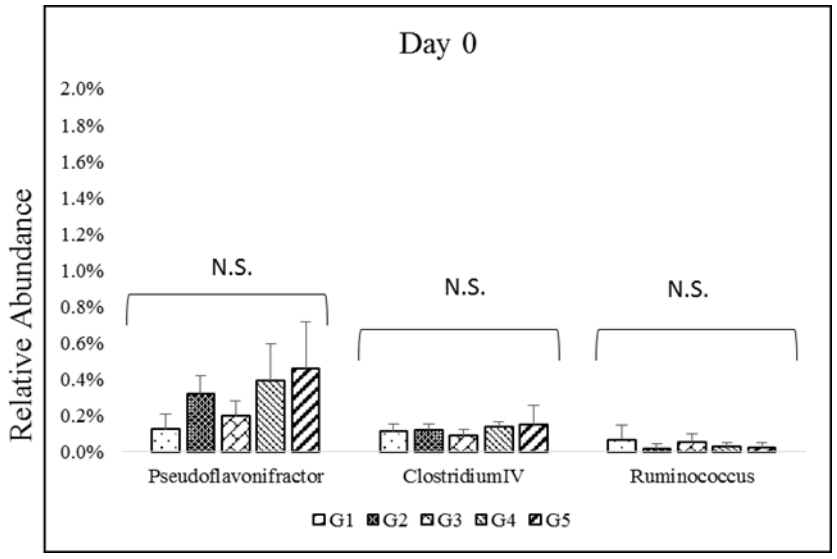
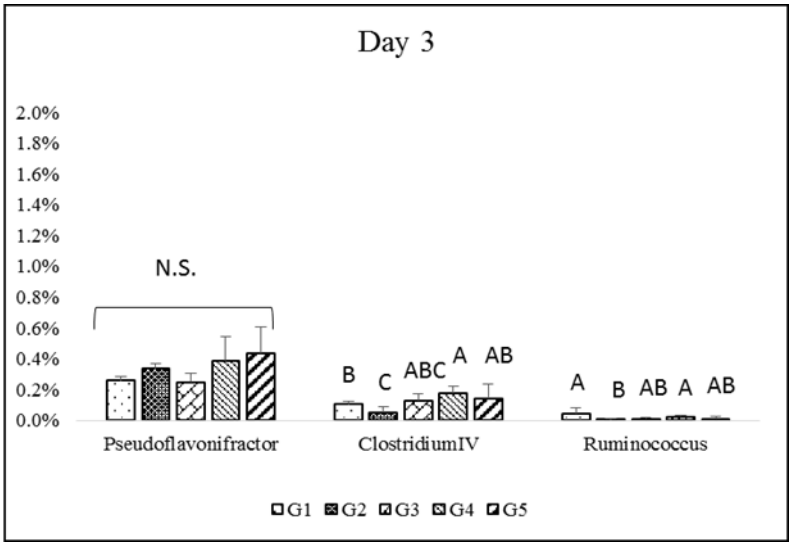
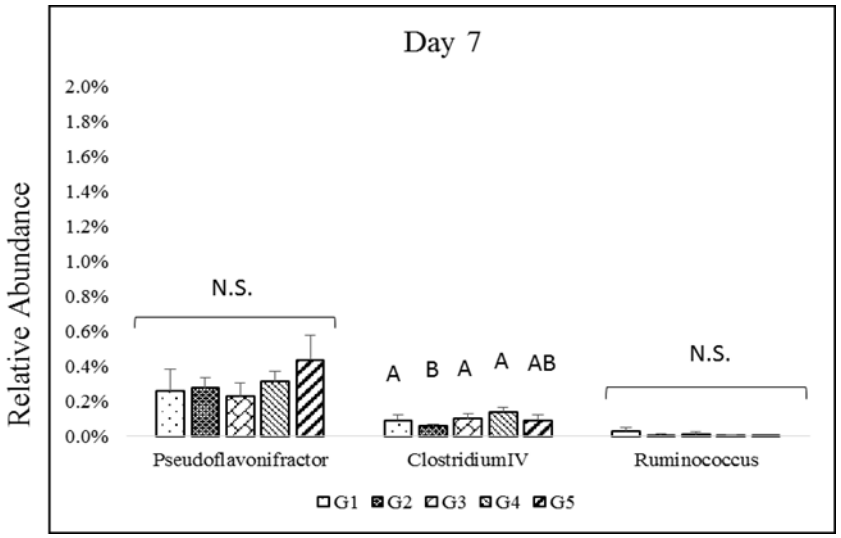



**Figure 4.5 D:** Impact of melanoidin malts on responsive genera relative abundance among the Firmicutes (Ruminococcaceae). Significant differences ($P<0.005$) are indicated by different letters.


Day 0
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
N.S.
N.S.
N.S.
Pseudoflavonifractor
ClostridiumIV
Ruminococcus
G1 G2 G3 G4 G5


Day 3
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
N.S.
B C ABC A AB
A B AB A AB
Pseudoflavonifractor
ClostridiumIV
Ruminococcus
G1 G2 G3 G4 G5


Day 7
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
N.S.
A B A A AB
N.S.
Pseudoflavonifractor
ClostridiumIV
Ruminococcus
G1 G2 G3 G4 G5

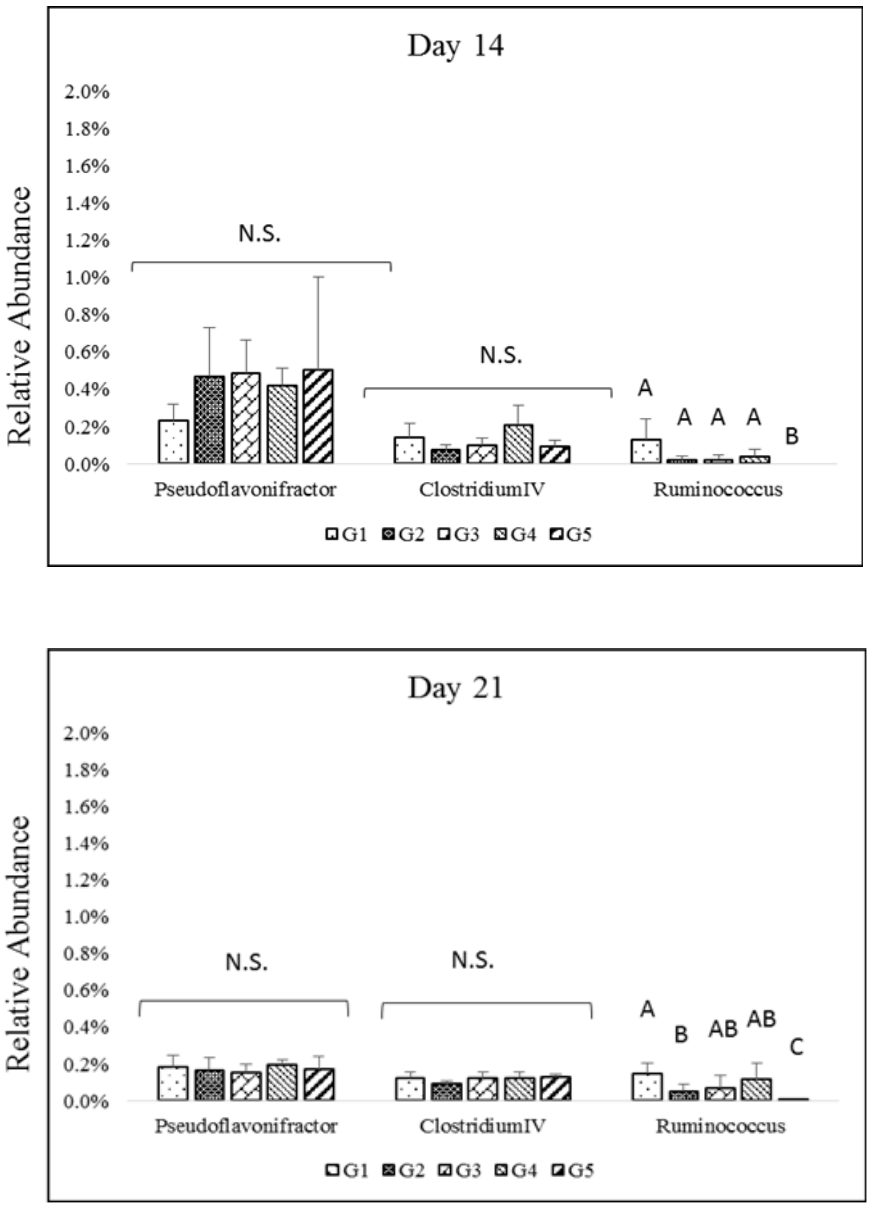
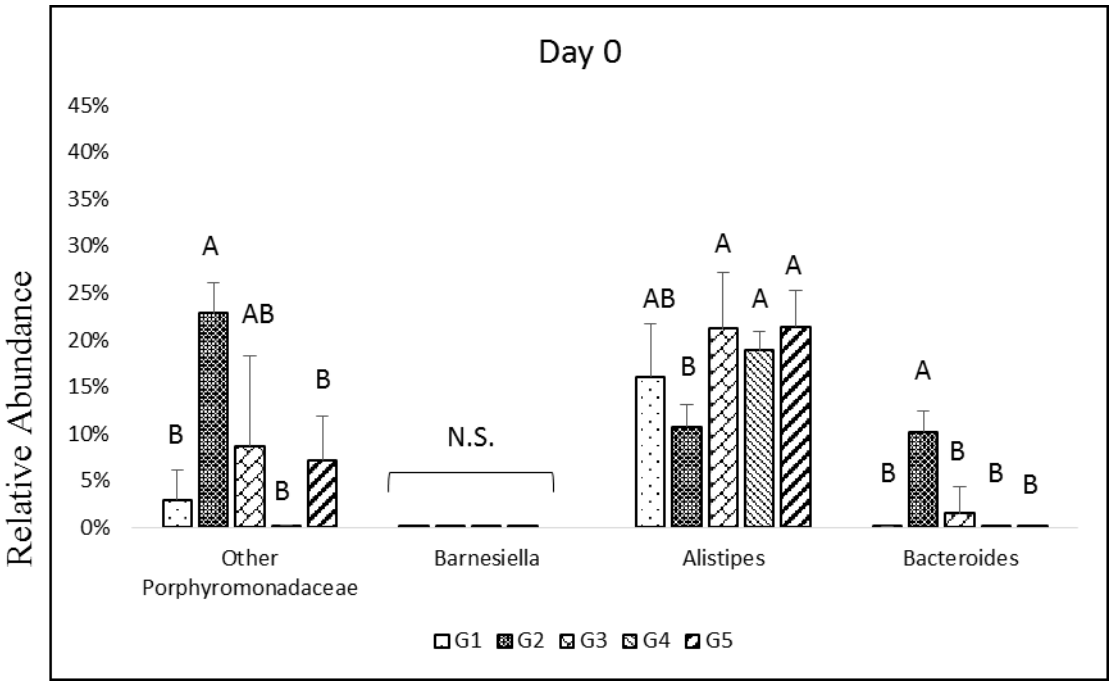
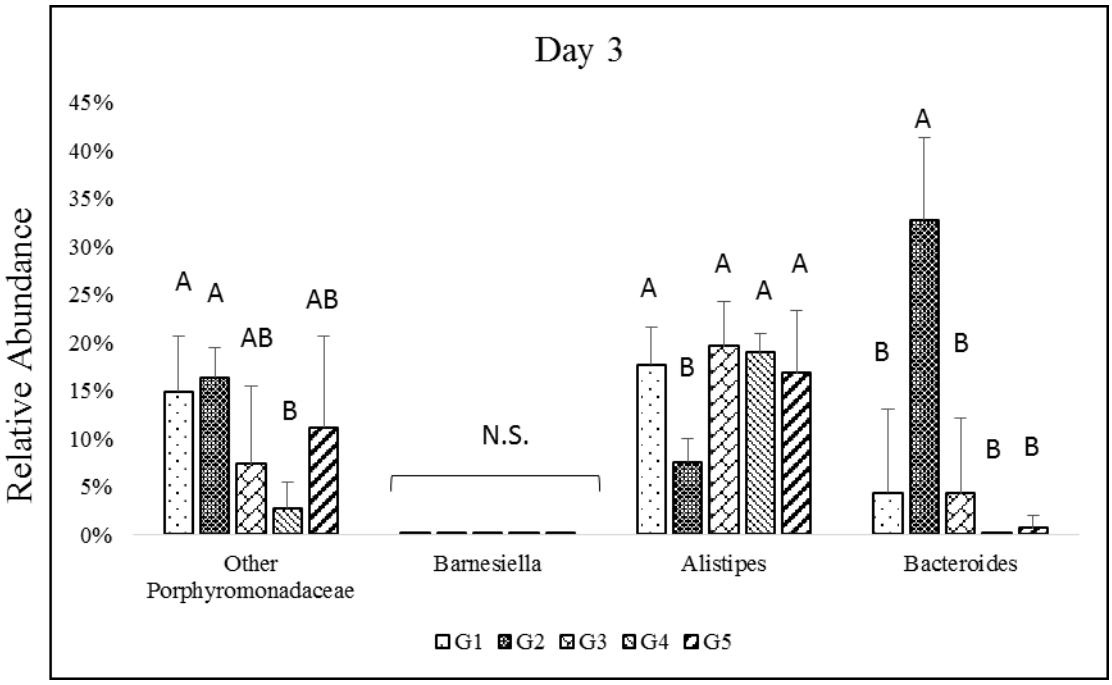
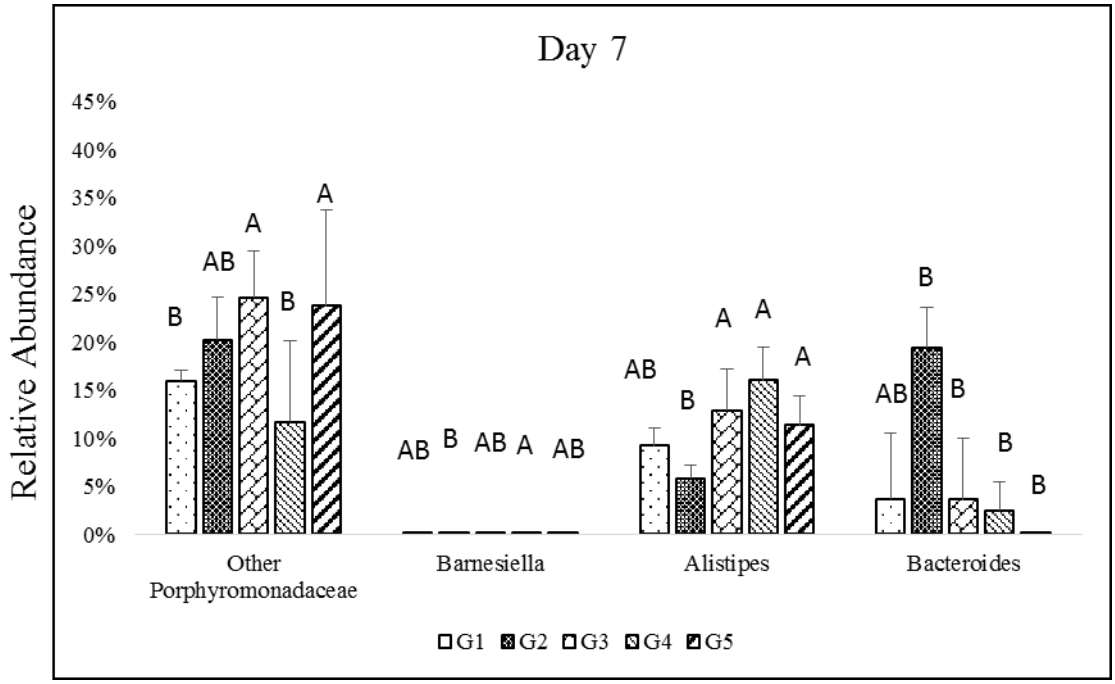
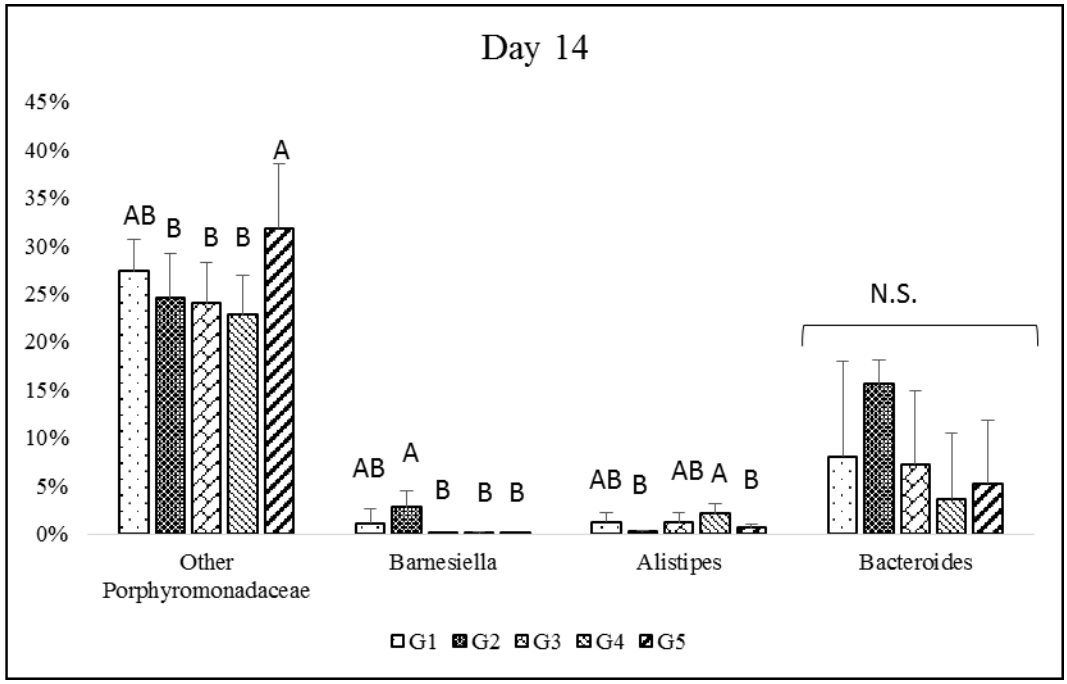



**Figure 4.5 E:** Impact of melanoidin malts on responsive genera relative abundance among the Firmicutes (Ruminococcaceae). Significant differences ($P<0.005$) are indicated by different letters.


Day 0
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
A
AB
B
B
N.S.
AB
B
A
A
A
B
A
B
B
B
Other Porphyromonadaceae
Barnesiella
Alistipes
Bacteroides
G1 G2 G3 G4 G5


Day 3
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
A
A
AB
B
AB
N.S.
A
B
A
A
A
B
A
B
B
B
Other Porphyromonadaceae
Barnesiella
Alistipes
Bacteroides
G1 G2 G3 G4 G5


Day 7
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
B
AB
A
B
A
AB
B
AB
A
AB
AB
B
A
A
A
AB
B
B
B
B
Other Porphyromonadaceae
Barnesiella
Alistipes
Bacteroides
G1 G2 G3 G4 G5


Day 14
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
AB
B
B
B
A
AB
A
B
B
B
AB
B
AB
A
B
N.S.
Other Porphyromonadaceae
Barnesiella
Alistipes
Bacteroides
G1 G2 G3 G4 G5

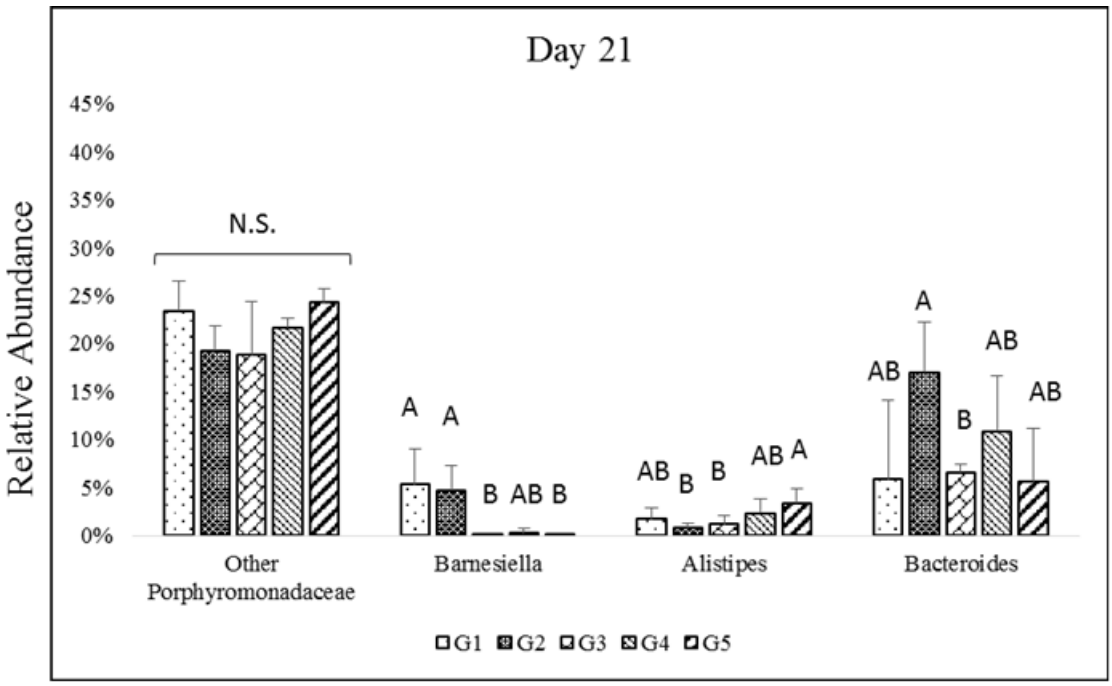
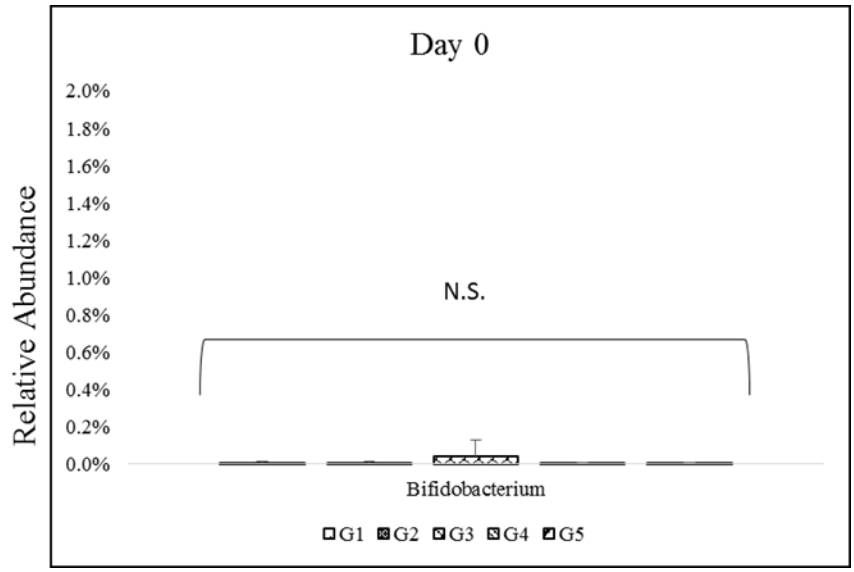
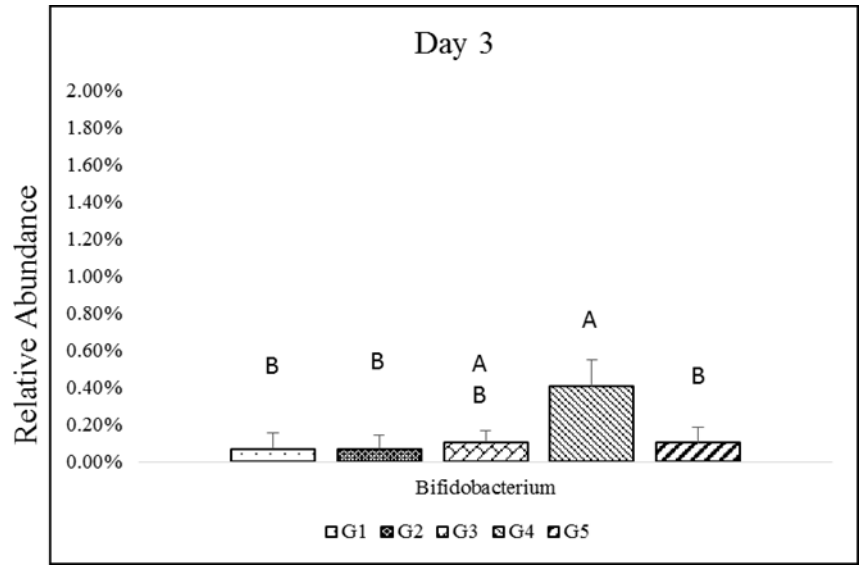
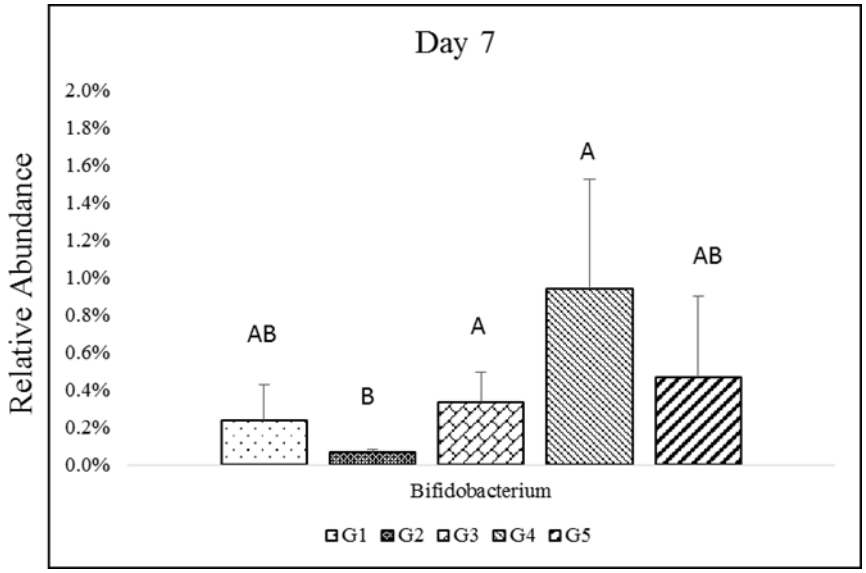



**Figure 4.6:** Impact of melanoidin malts on responsive genera relative abundance among the Bacteroidetes. Significant differences ($P$<0.005) are indicated by different letters.


Day 0
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
N.S.
Bifidobacterium
G1 G2 G3 G4 G5


Day 3
Relative Abundance
2.00%
1.80%
1.60%
1.40%
1.20%
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
B
B
A
B
A
B
Bifidobacterium
G1 G2 G3 G4 G5


Day 7
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
AB
B
A
A
AB
Bifidobacterium
G1 G2 G3 G4 G5

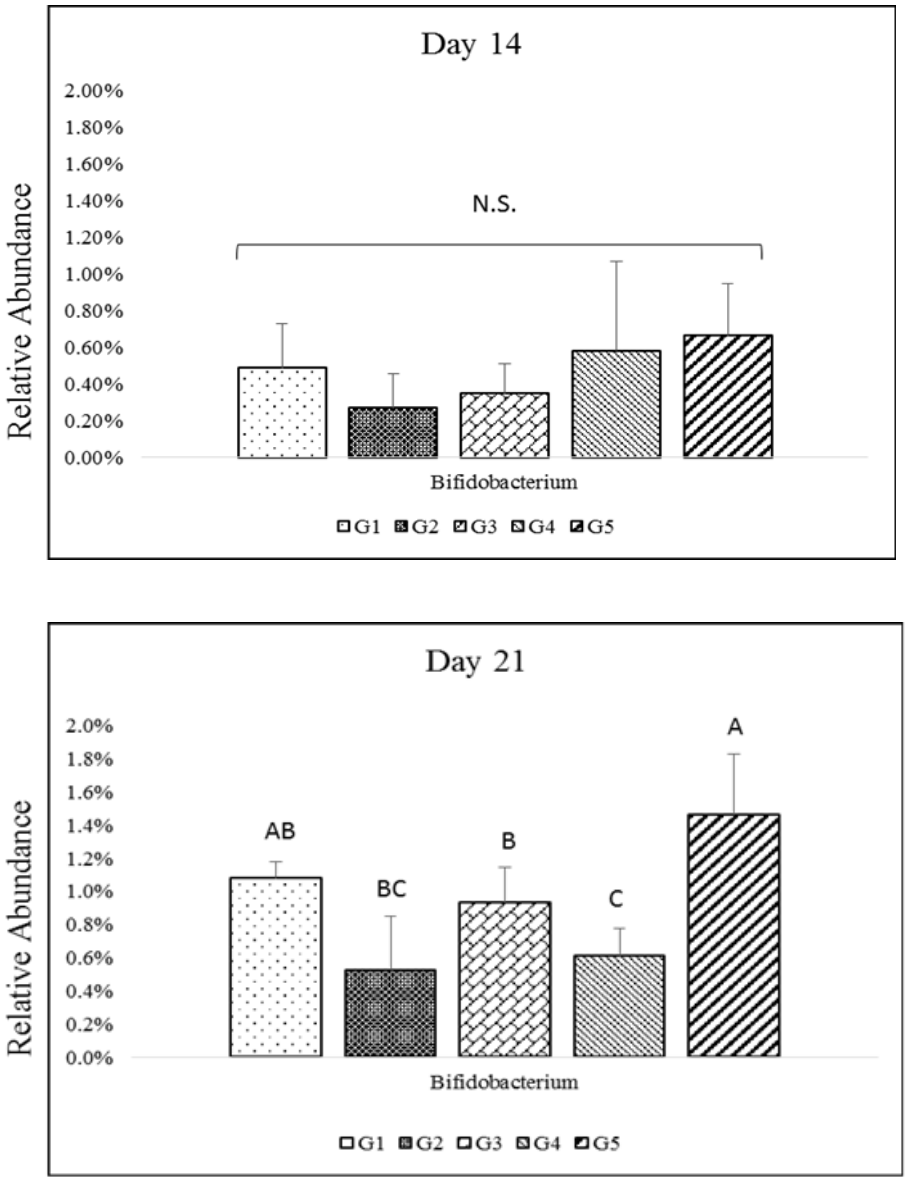
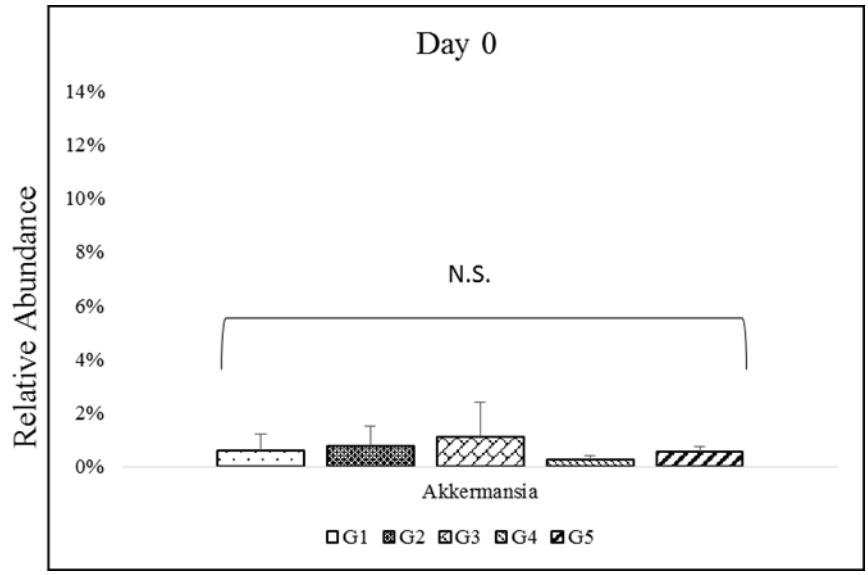
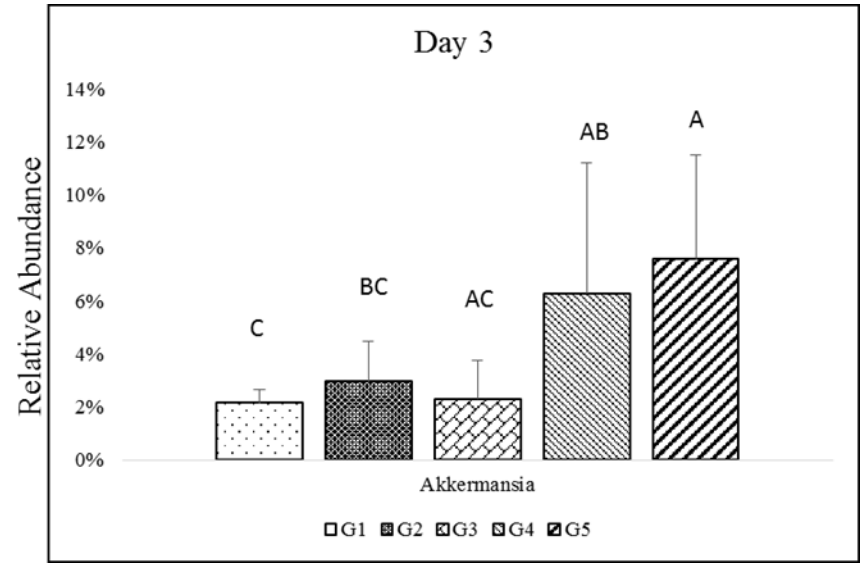
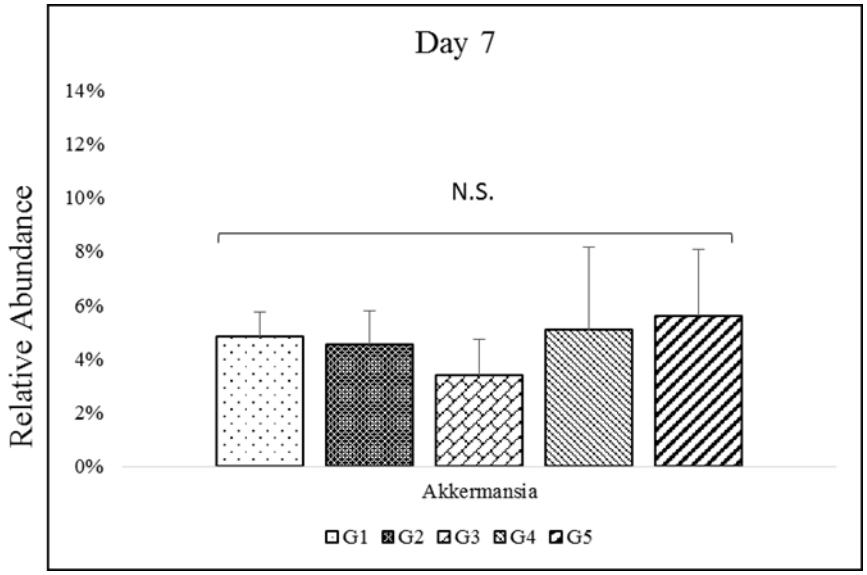



**Figure 4.7 A:** Impact of melanoidin malts on responsive genera relative abundance among the Actinobacteria phyla (*Bifidobacterium*). Significant differences ($P<0.005$) are indicated by different letters.


Day 0
14%
12%
10%
8%
6%
4%
2%
0%
Relative Abundance
N.S.
Akkermansia
G1 G2 G3 G4 G5


Day 3
14%
12%
10%
8%
6%
4%
2%
0%
Relative Abundance
C
BC
AC
AB
A
Akkermansia
G1 G2 G3 G4 G5


Day 7
14%
12%
10%
8%
6%
4%
2%
0%
Relative Abundance
N.S.
Akkermansia
G1 G2 G3 G4 G5

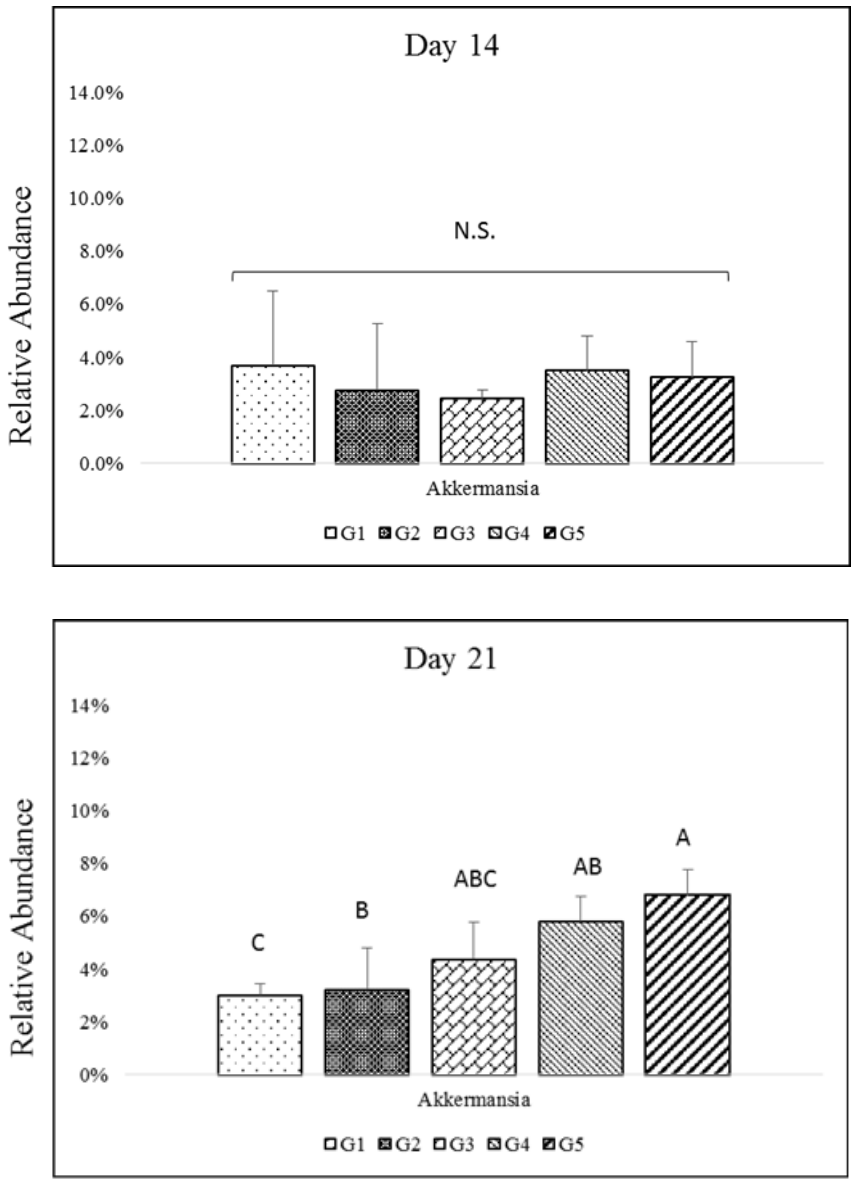
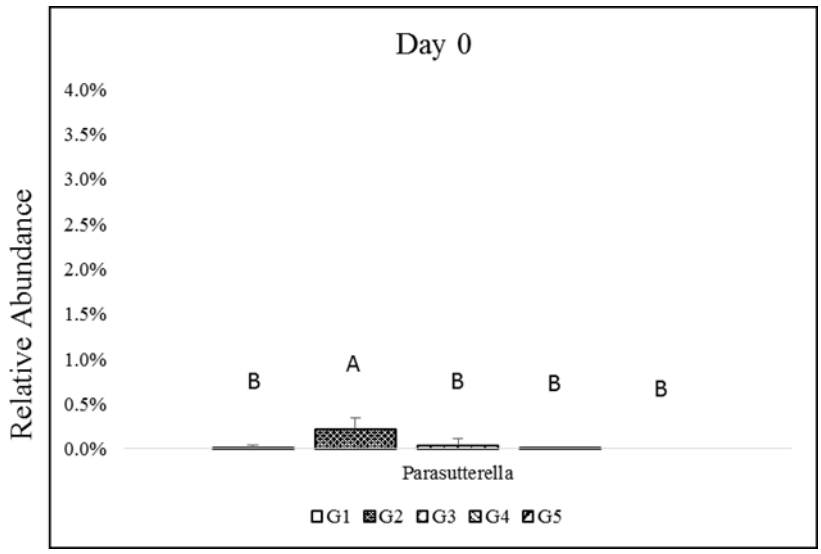
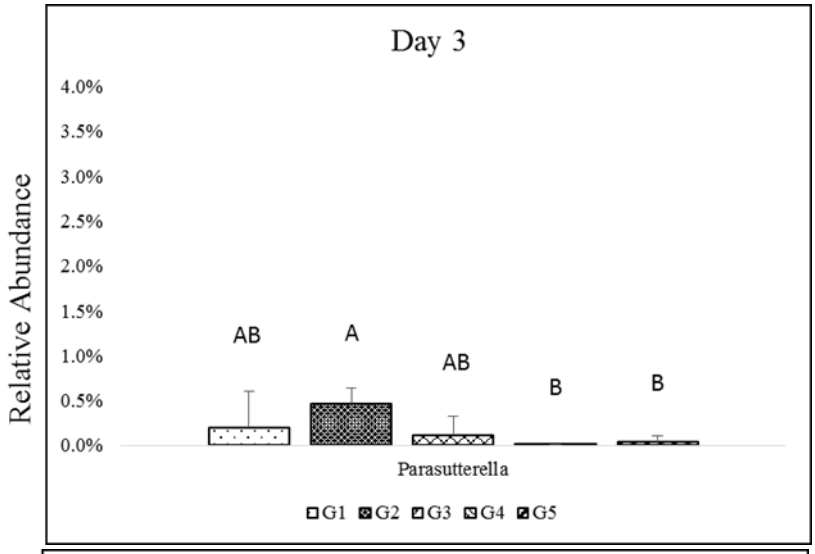
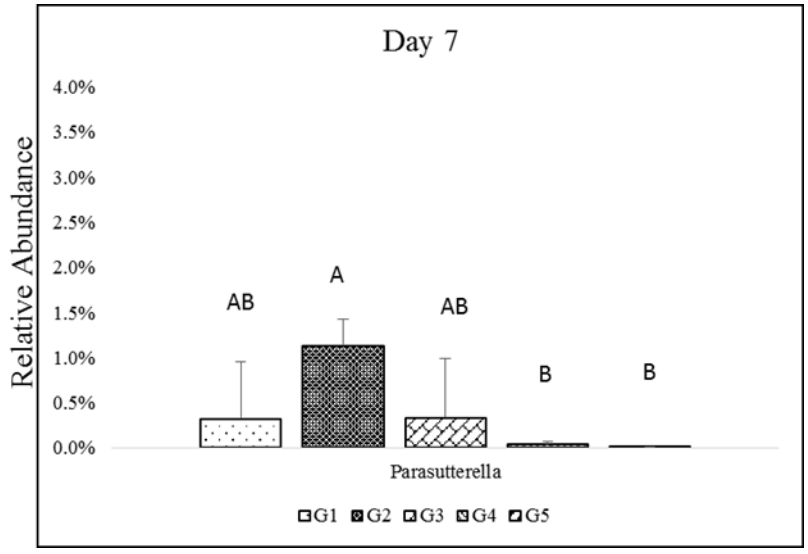



**Figure 4.7 B:** Impact of melanoidin malts on responsive genera relative abundance among the Verrumicrobia phyla (*Akkermansia*). Significant differences ($P$<0.005) are indicated by different letters.


Day 0
Relative Abundance
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
B
A
B
B
B
Parasutterella
G1 G2 G3 G4 G5


Day 3
Relative Abundance
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
AB
A
AB
B
B
Parasutterella
G1 G2 G3 G4 G5


Day 7
Relative Abundance
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
AB
A
AB
B
B
Parasutterella
G1 G2 G3 G4 G5

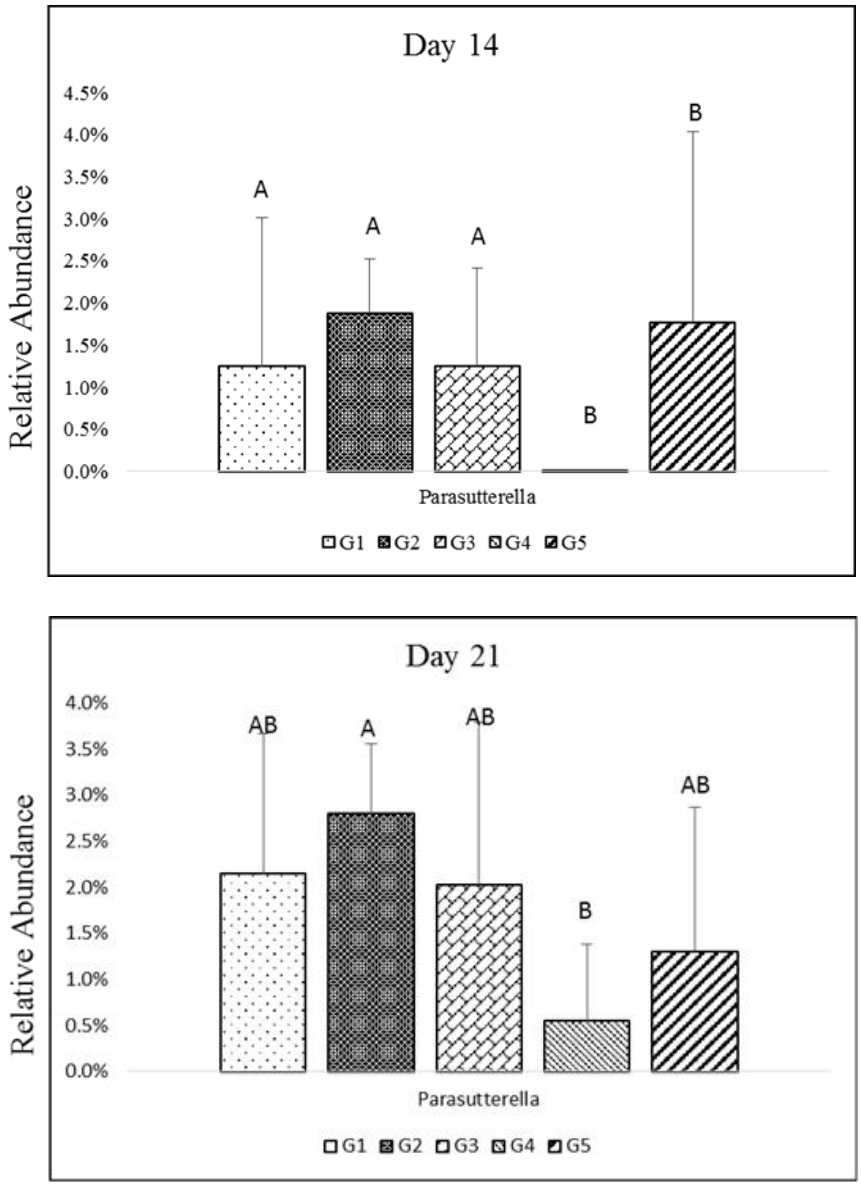
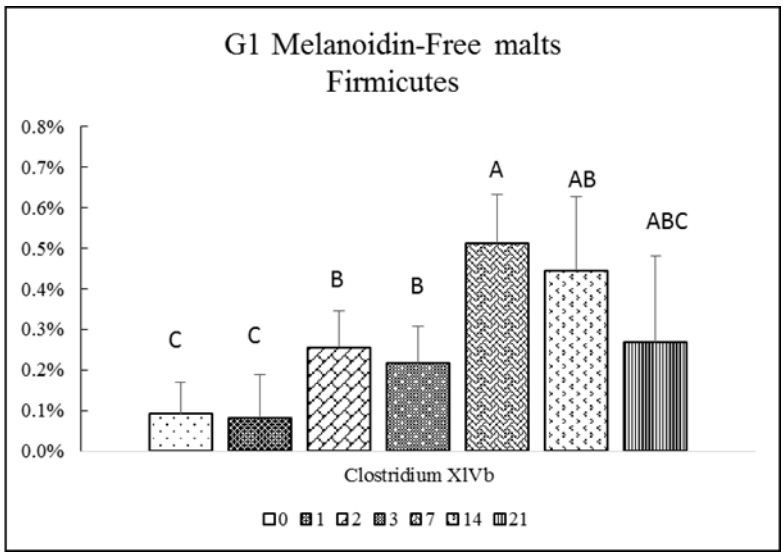
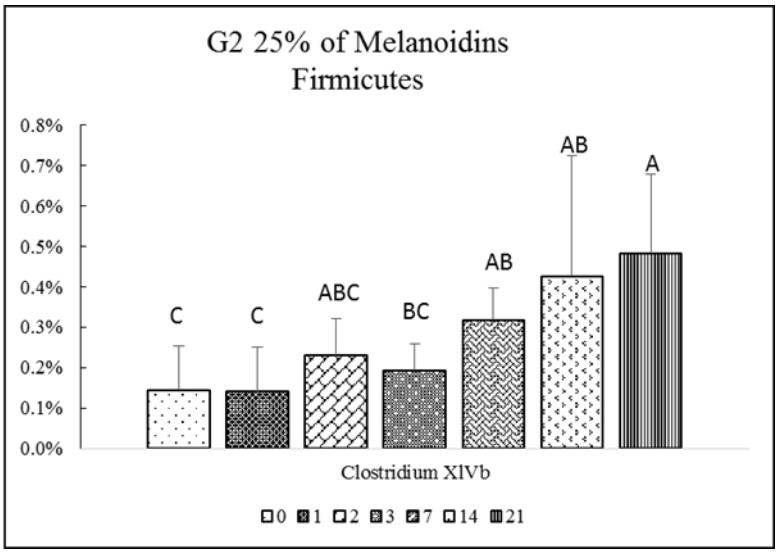
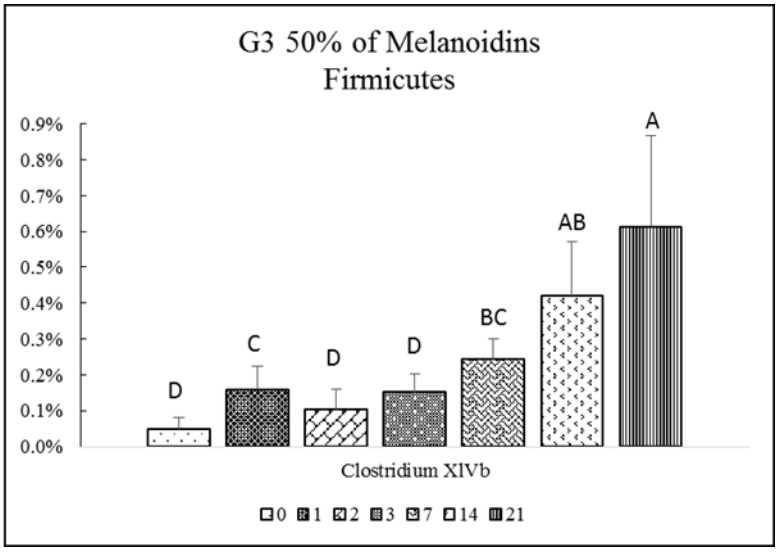



**Figure 4.7 C**: Impact of melanoidin malts on responsive genera relative abundance among the Proteobacteria phyla (*Parasutturella*). Significant differences ($P$<0.005) are indicated by different letters.


G1 Melanoidin-Free malts
Firmicutes
Relative Abundance
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
C
C
B
B
A
AB
ABC
Clostridium XlVb
0 1 2 3 7 14 21


G2 25% of Melanoidins
Firmicutes
Relative Abundance
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
C
C
ABC
BC
AB
AB
A
Clostridium XlVb
0 1 2 3 7 14 21


G3 50% of Melanoidins
Firmicutes
Relative Abundance
0.9%
0.8%
0.7%
0.6%
0.5%
0.4%
0.3%
0.2%
0.1%
0.0%
D
C
D
D
BC
AB
A
Clostridium XlVb
0 1 2 3 7 14 21

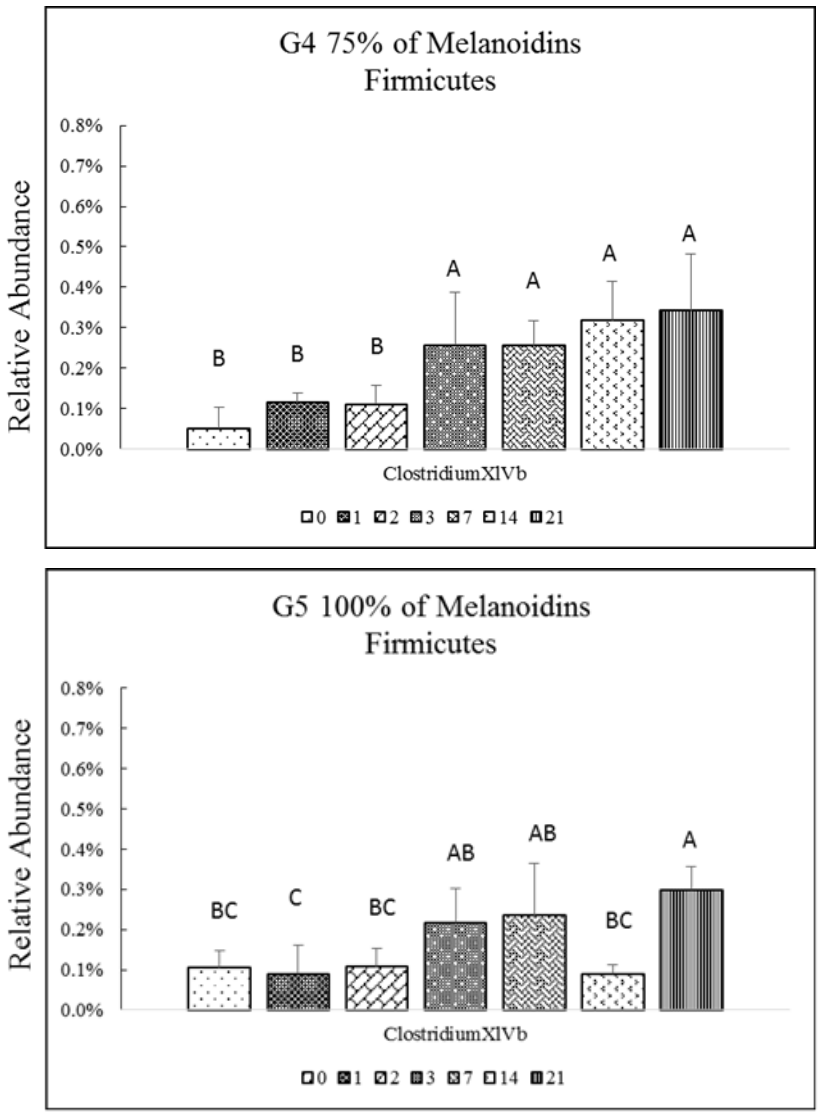
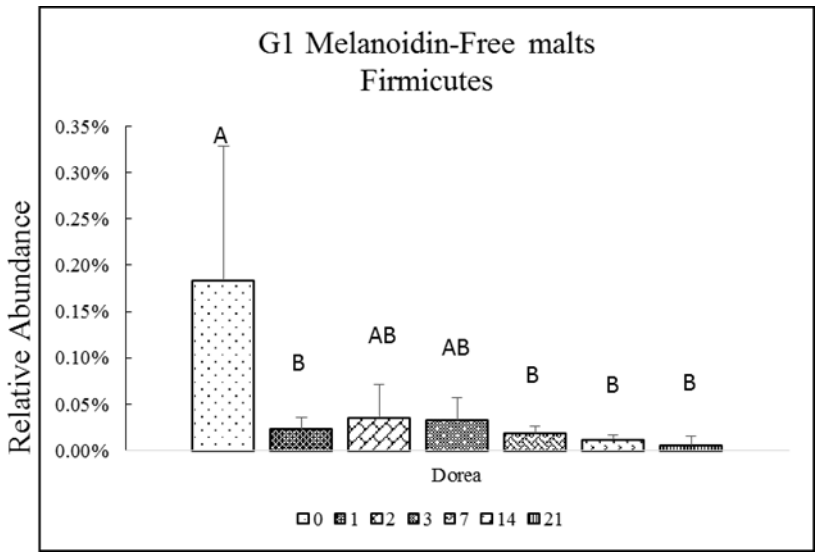
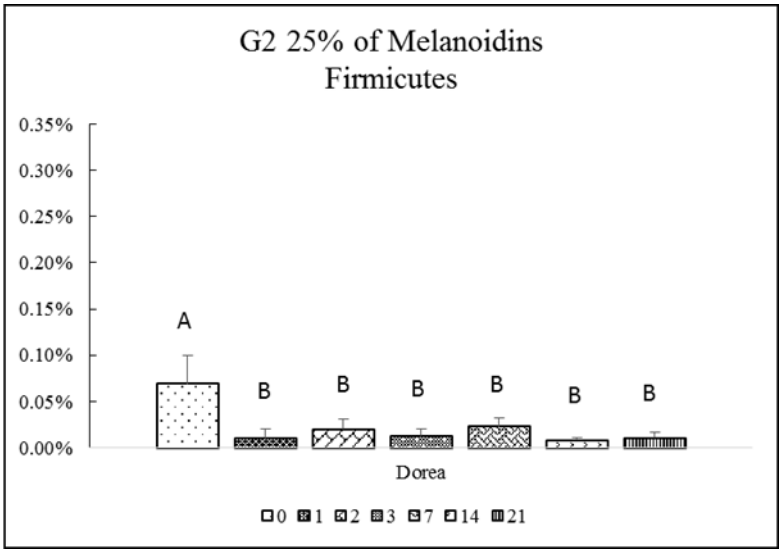
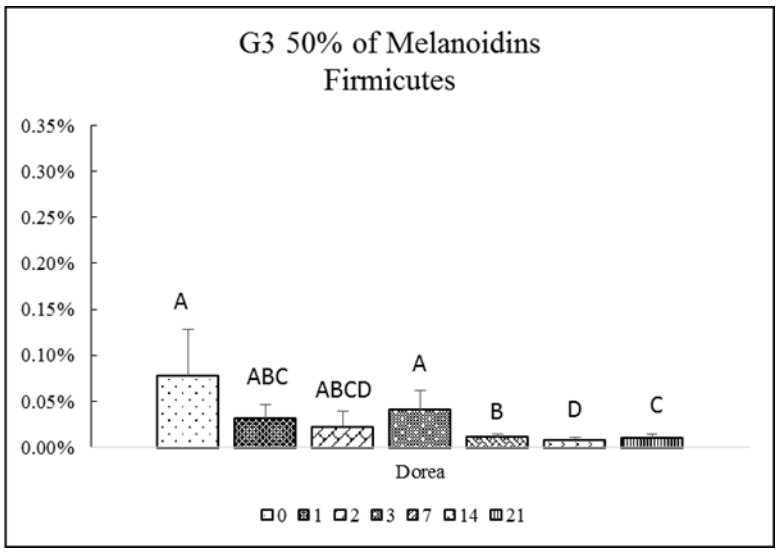



**Figure 4.8 A**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Firmicutes phyla (*ClostridiumXlVb*) throughout study. Significant difference ($P$<0.005) are indicated by different letters.


G1 Melanoidin-Free malts
Firmicutes
Relative Abundance
0.35%
0.30%
0.25%
0.20%
0.15%
0.10%
0.05%
0.00%
A
B
AB
AB
B
B
B
Dorea
0 1 2 3 7 14 21


G2 25% of Melanoidins
Firmicutes
Relative Abundance
0.35%
0.30%
0.25%
0.20%
0.15%
0.10%
0.05%
0.00%
A
B
B
B
B
B
B
Dorea
0 1 2 3 7 14 21


G3 50% of Melanoidins
Firmicutes
Relative Abundance
0.35%
0.30%
0.25%
0.20%
0.15%
0.10%
0.05%
0.00%
A
ABC
ABCD
A
B
D
C
Dorea
0 1 2 3 7 14 21

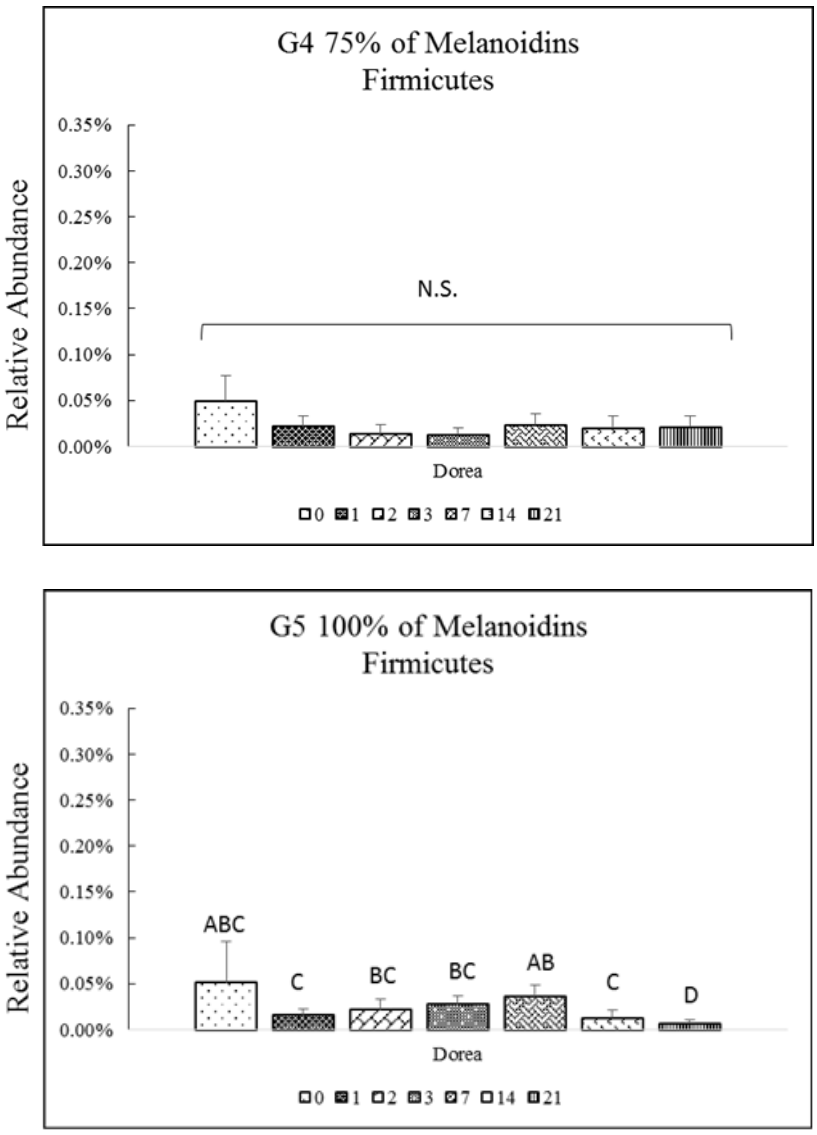
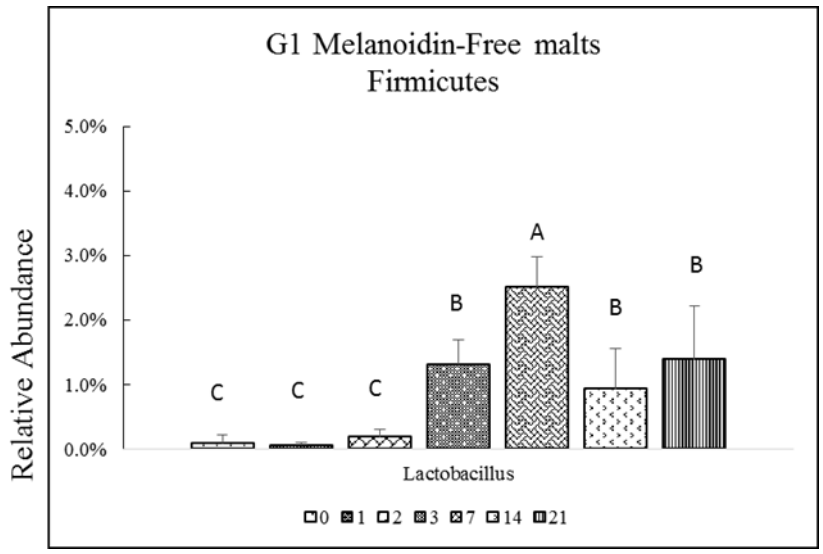
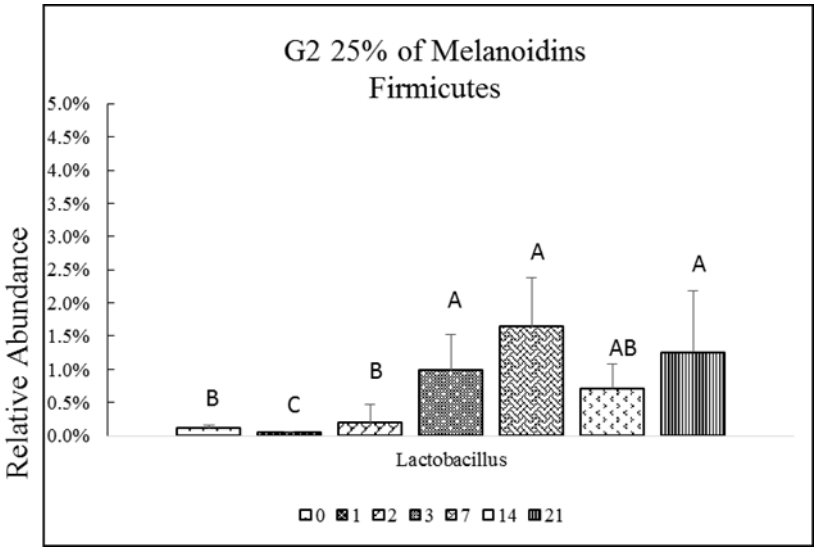
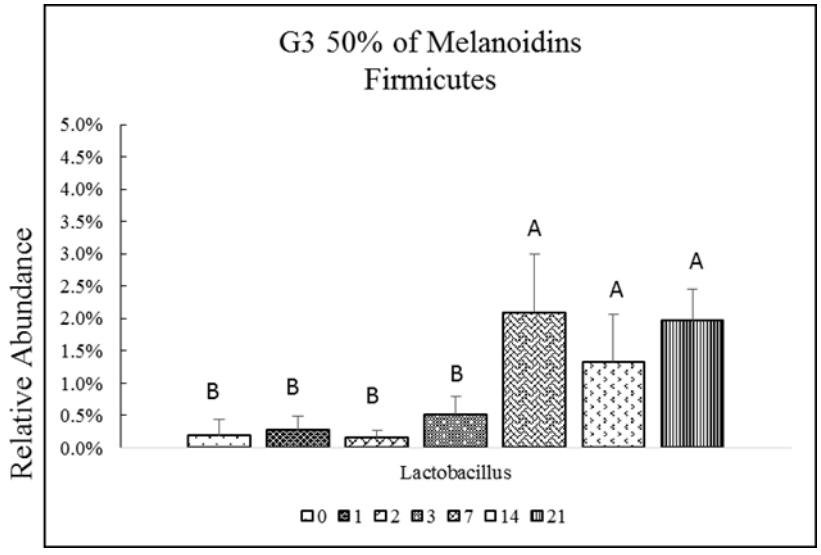
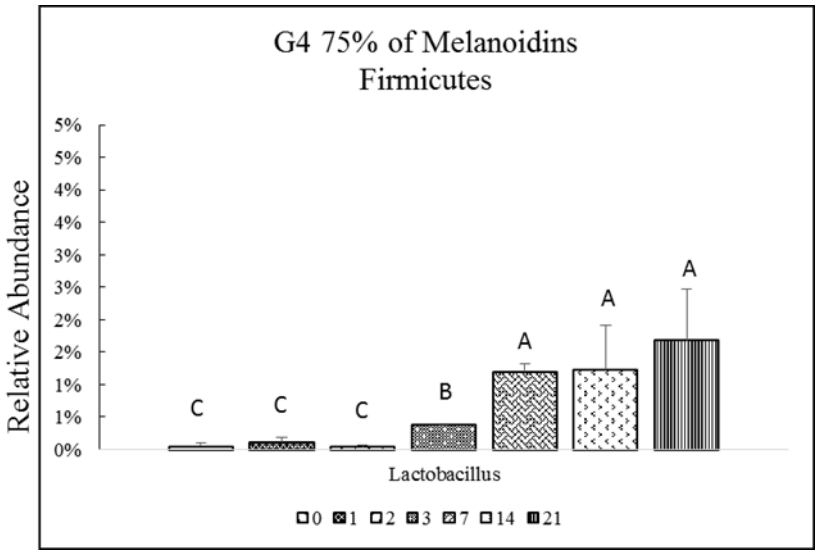



**Figure 4.8 B**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Firmicutes phyla (*Dorea*) throughout study. Significant difference ($P$<0.005) are indicated by different letters.


G1 Melanoidin-Free malts
Firmicutes
Relative Abundance
5.0%
4.0%
3.0%
2.0%
1.0%
0.0%
C
C
C
B
A
B
B
Lactobacillus
0 1 2 3 7 14 21


G2 25% of Melanoidins
Firmicutes
Relative Abundance
5.0%
4.5%
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
B
C
B
A
A
AB
A
Lactobacillus
0 1 2 3 7 14 21


G3 50% of Melanoidins
Firmicutes
Relative Abundance
5.0%
4.5%
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
B
B
B
B
A
A
A
Lactobacillus
0 1 2 3 7 14 21



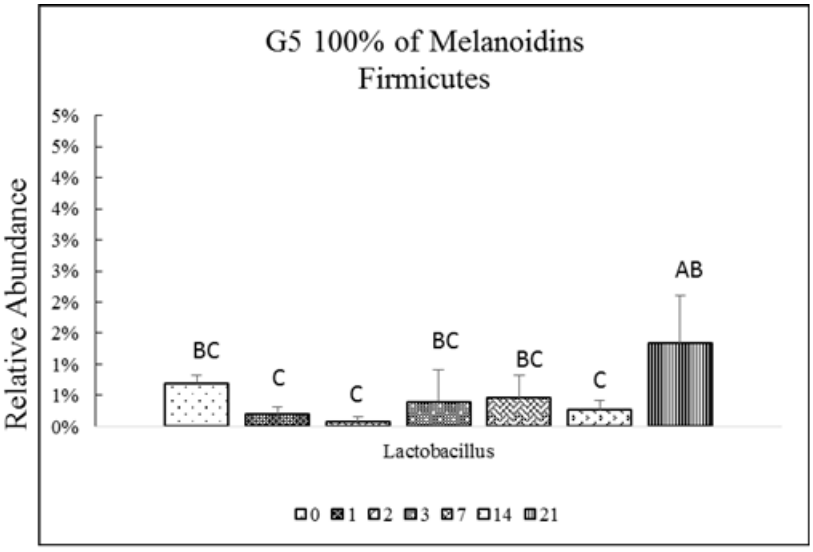
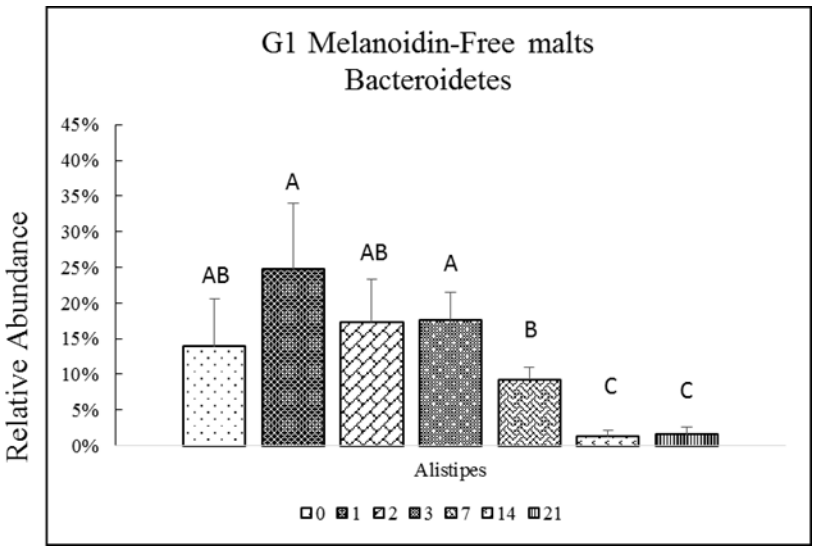
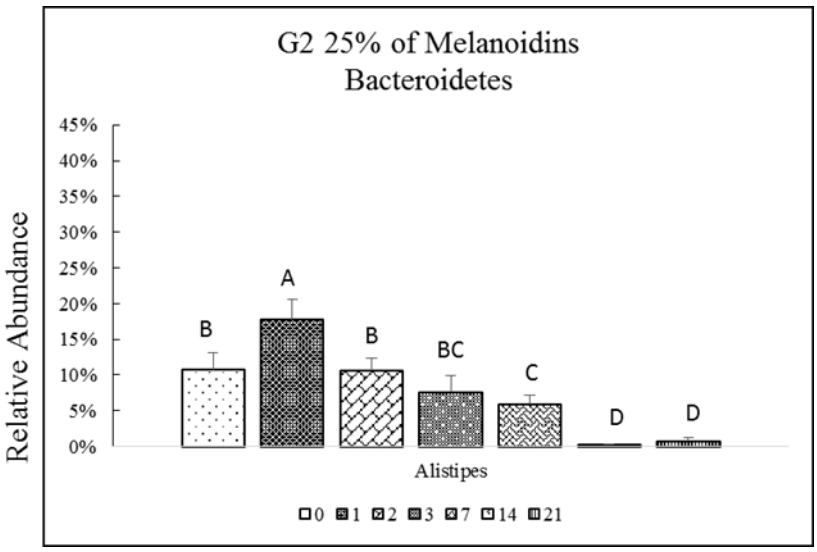
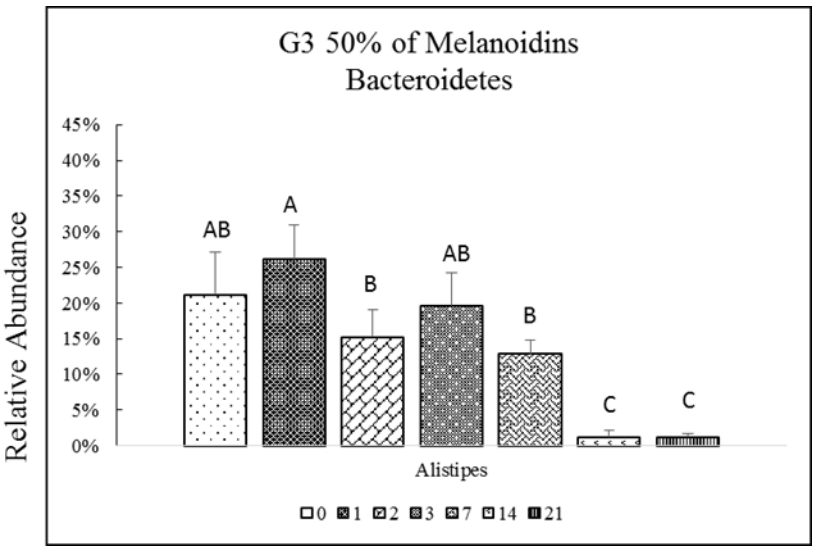
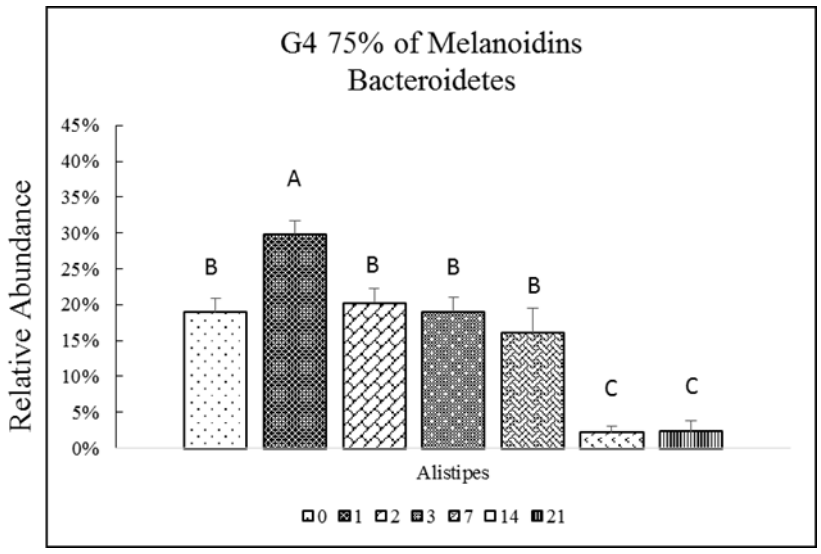




**Figure 4.8 C**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Firmicutes phyla (*Lactobacillus*) throughout study. Significant difference ($P$<0.005) are indicated by different letters.


G1 Melanoidin-Free malts
Bacteroidetes
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
AB
A
AB
A
B
C
C
Alistipes
0 1 2 3 7 14 21


G2 25% of Melanoidins
Bacteroidetes
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
B
A
B
BC
C
D
D
Alistipes
0 1 2 3 7 14 21


G3 50% of Melanoidins
Bacteroidetes
Relative Abundance
45%
40%
35%
30%
25%
20%
15%
10%
5%
0%
AB
A
B
AB
B
C
C
Alistipes
0 1 2 3 7 14 21



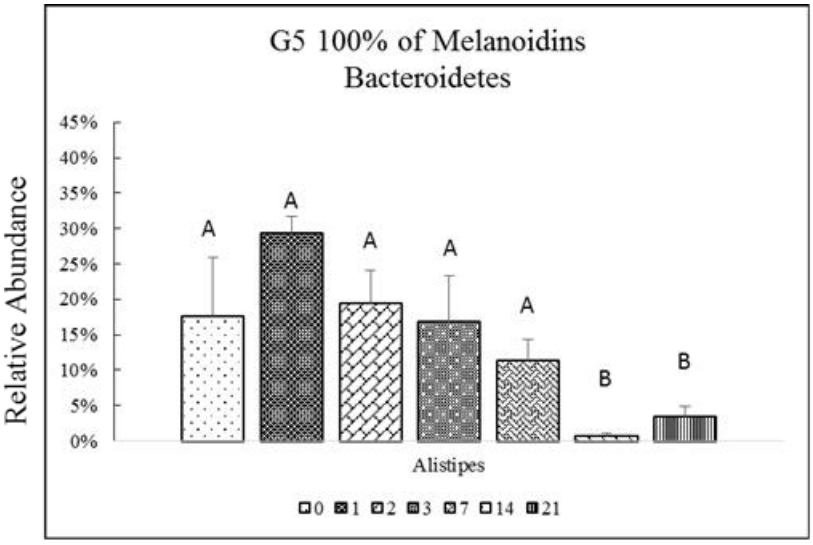
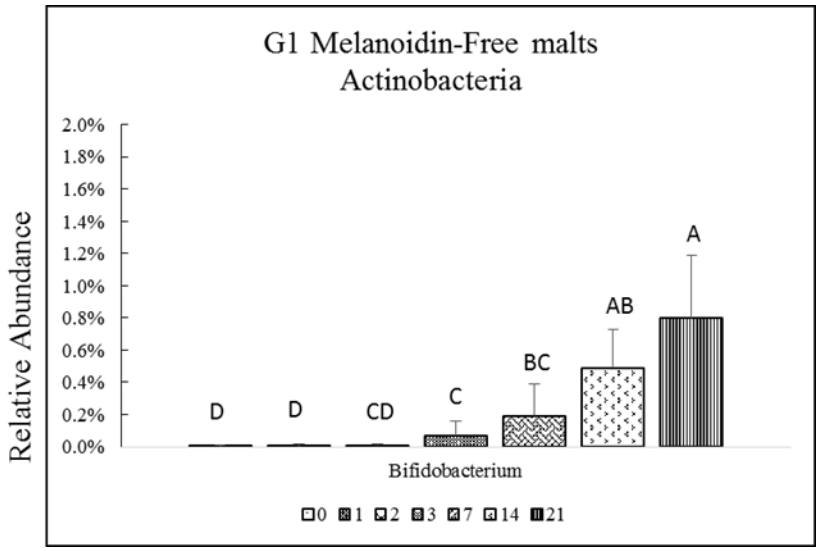
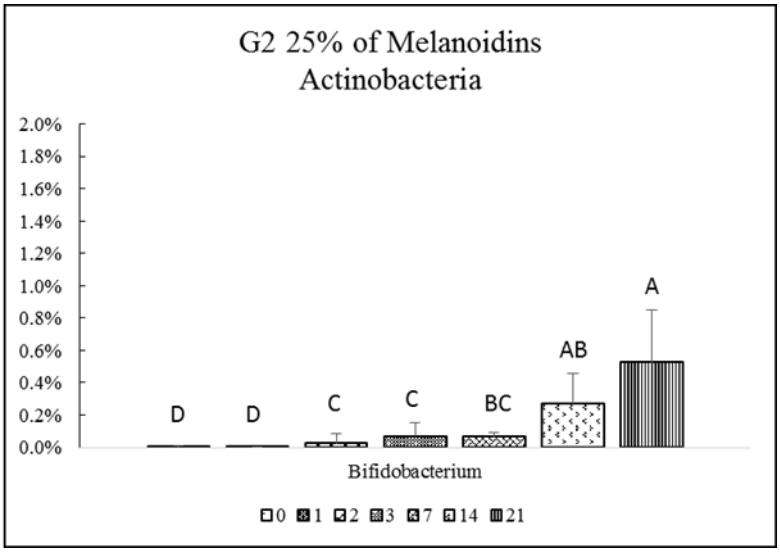
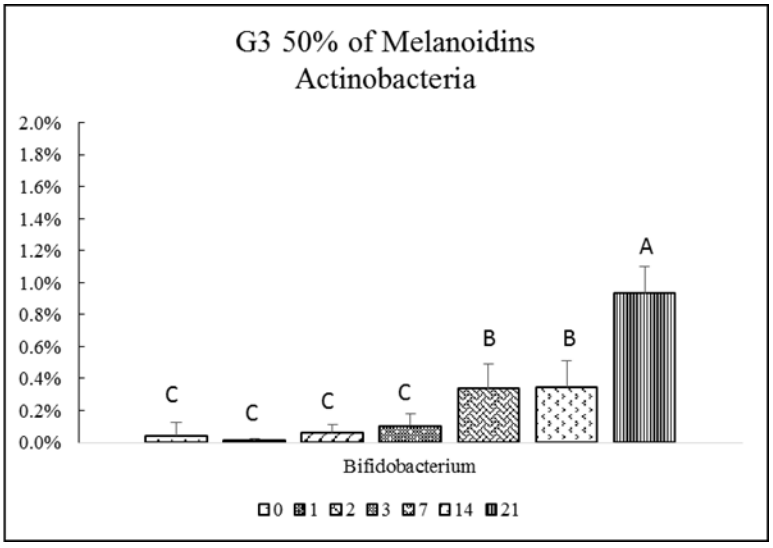




**Figure 4.8 D**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Bacteroidetes phyla (*Alistipes*) throughout study. Significant difference ($P$<0.005) are indicated by different letters.


G1 Melanoidin-Free malts
Actinobacteria
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
D
D
CD
C
BC
AB
A
Bifidobacterium
0 1 2 3 7 14 21


G2 25% of Melanoidins
Actinobacteria
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
D
D
C
C
BC
AB
A
Bifidobacterium
0 1 2 3 7 14 21


G3 50% of Melanoidins
Actinobacteria
Relative Abundance
2.0%
1.8%
1.6%
1.4%
1.2%
1.0%
0.8%
0.6%
0.4%
0.2%
0.0%
C
C
C
C
B
B
A
Bifidobacterium
0 1 2 3 7 14 21

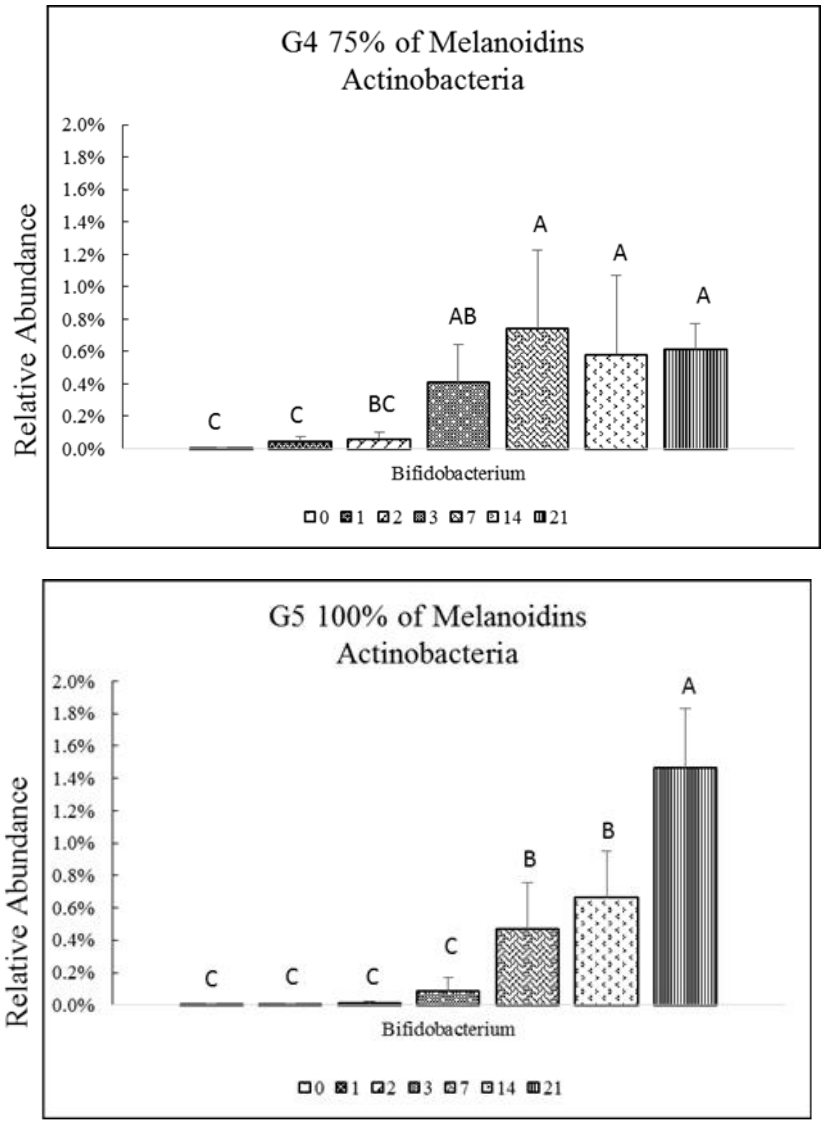
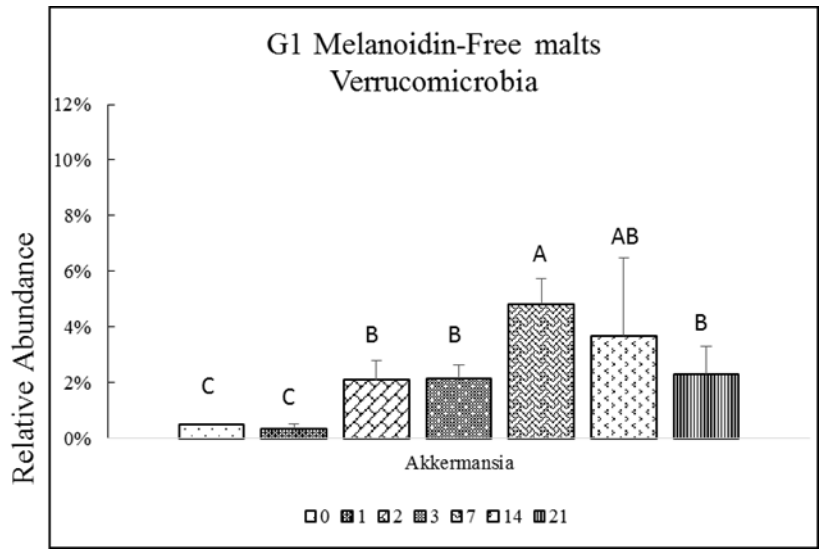
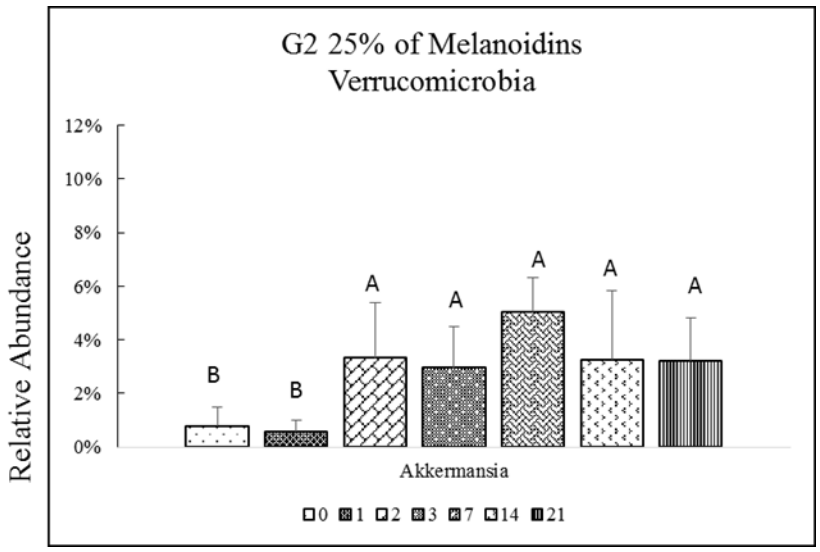
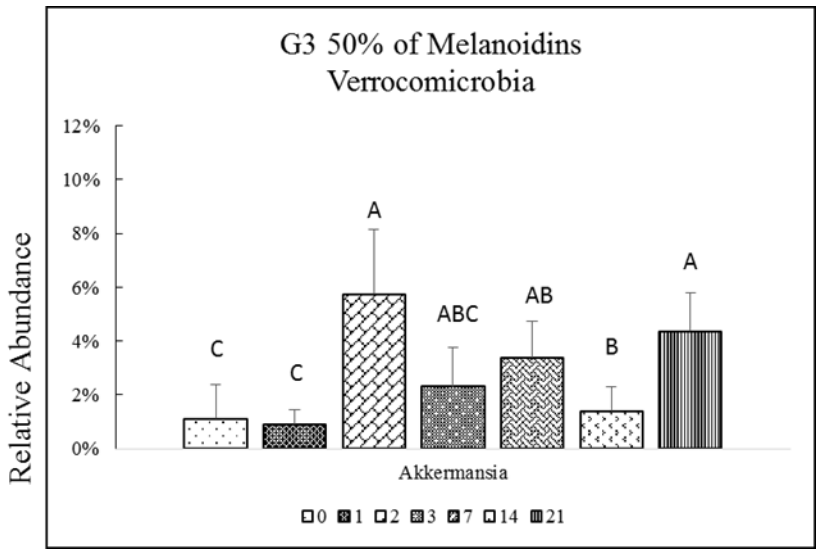



**Figure 4.8 E**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Actinobacteria phyla (*Bifidobacteria*) throughout study. Significant difference ($P$<0.005) are indicated by different letters.


G1 Melanoidin-Free malts
Verrucomicrobia
Relative Abundance
12%
10%
8%
6%
4%
2%
0%
C
C
B
B
A
AB
B
Akkermansia
0 1 2 3 7 14 21


G2 25% of Melanoidins
Verrucomicrobia
Relative Abundance
12%
10%
8%
6%
4%
2%
0%
B
B
A
A
A
A
A
Akkermansia
0 1 2 3 7 14 21


G3 50% of Melanoidins
Verrocomicrobia
Relative Abundance
12%
10%
8%
6%
4%
2%
0%
C
C
A
ABC
AB
B
A
Akkermansia
0 1 2 3 7 14 21

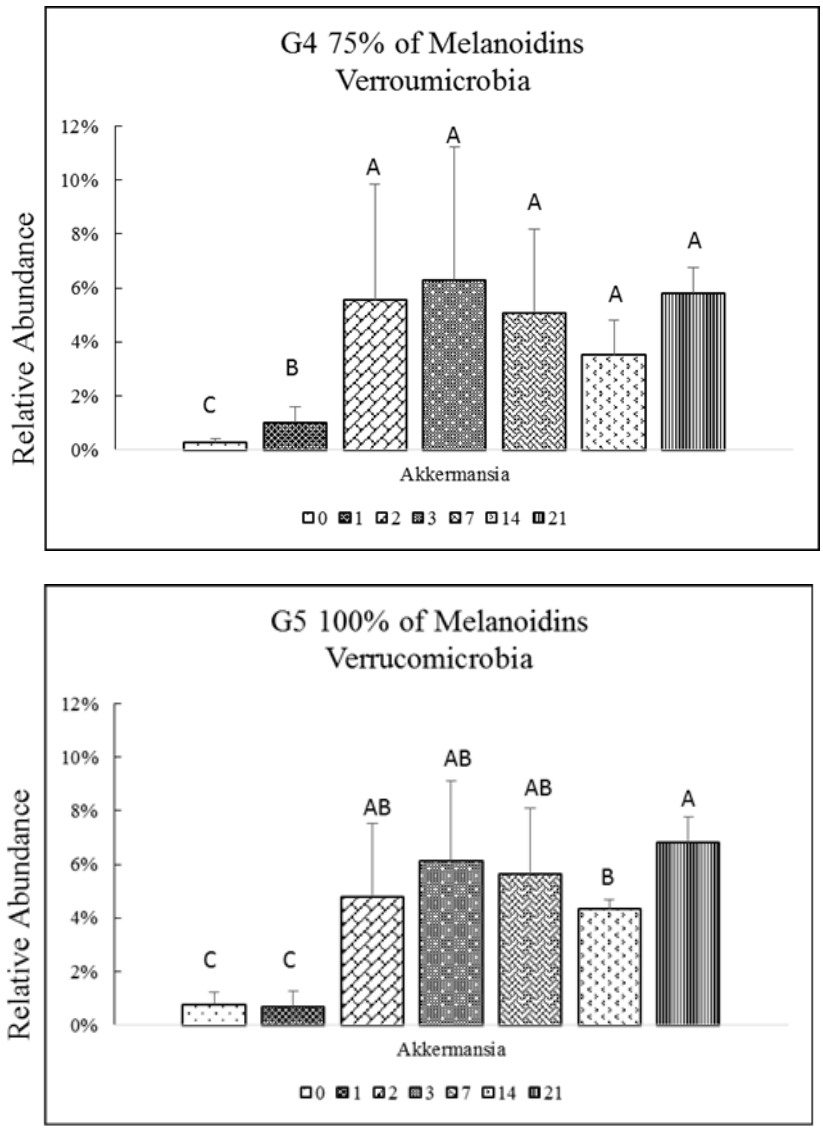
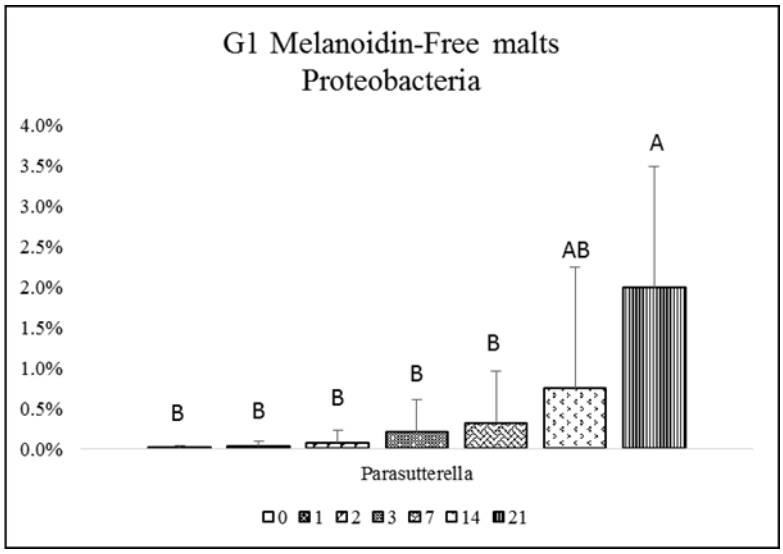
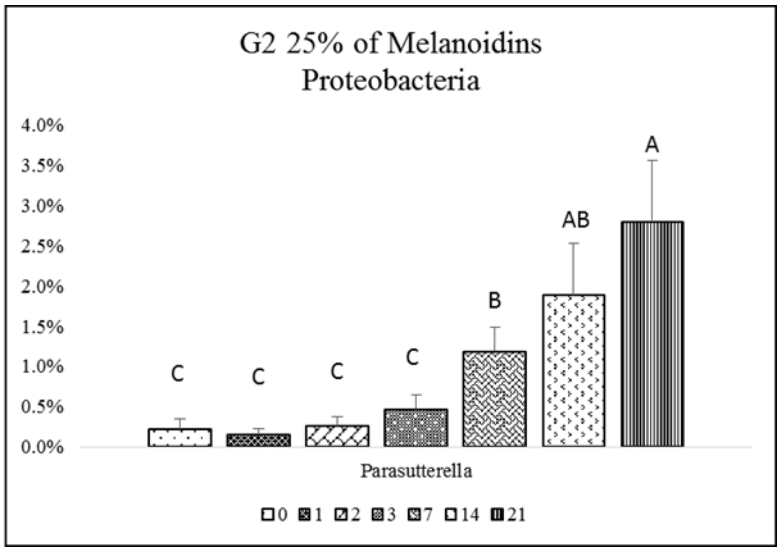
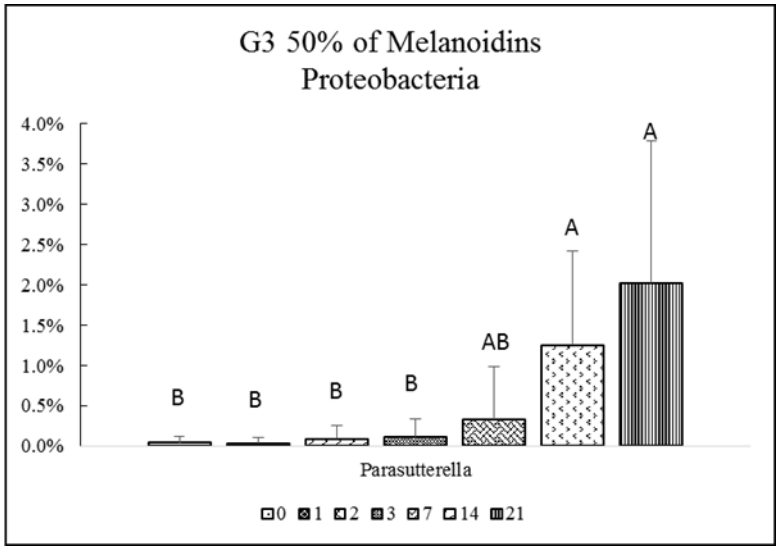



**Figure 4.8 F**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Verrucomicrobia phyla (*Akkermansia*) throughout study. Significant difference ($P<0.005$) are indicated by different letters.


G1 Melanoidin-Free malts
Proteobacteria
Relative Abundance
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
B
B
B
B
B
AB
A
Parasutterella
0 1 2 3 7 14 21


G2 25% of Melanoidins
Proteobacteria
Relative Abundance
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
C
C
C
C
B
AB
A
Parasutterella
0 1 2 3 7 14 21


G3 50% of Melanoidins
Proteobacteria
Relative Abundance
4.0%
3.5%
3.0%
2.5%
2.0%
1.5%
1.0%
0.5%
0.0%
B
B
B
B
AB
A
A
Parasutterella
0 1 2 3 7 14 21

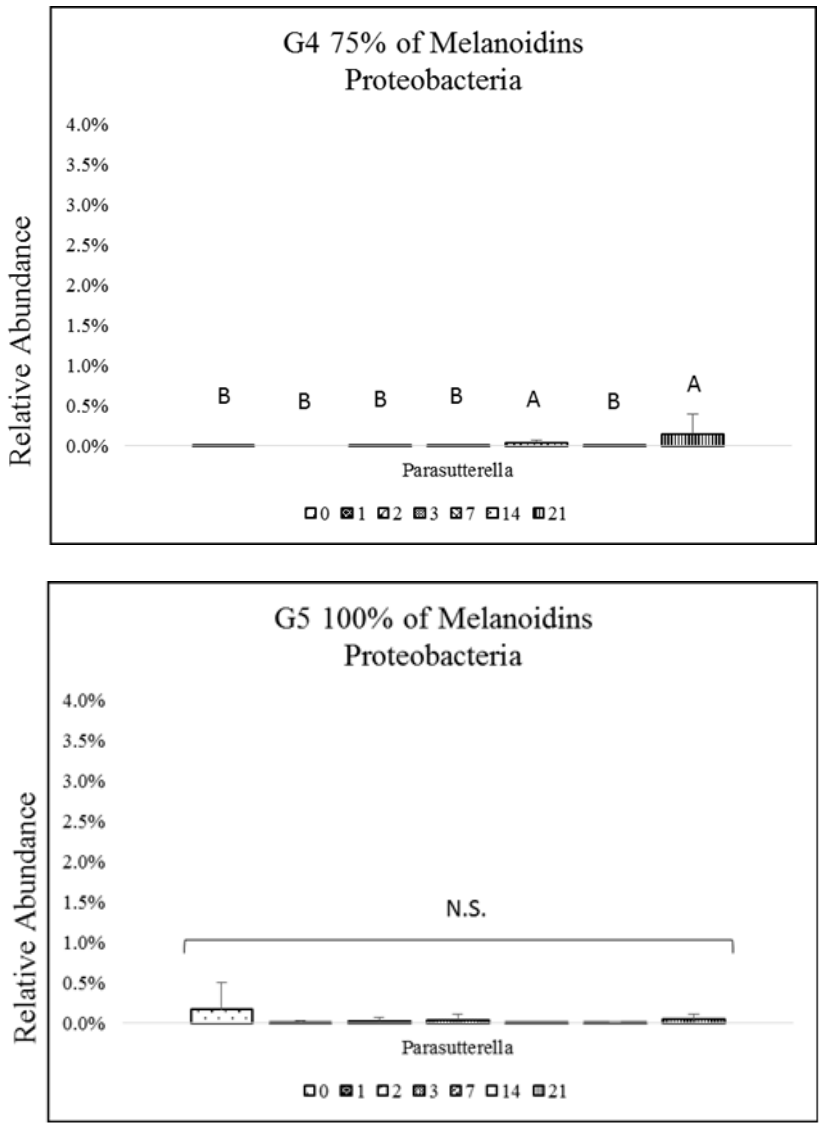



**Figure 4.8 G**: Impact of different portions of dietary melanoidin malts on responsive genera relative abundance among Proteobacteria phyla (*Parasutterella*) throughout study. Significant difference ($P$<0.005) are indicated by different letters.

GENERAL CONCLUSION:

Gut microbiota plays an important role in maintaining human health, providing energy for hosts, and developing the immune system. Considerable attention has been dedicated to the study of the impact diet on gut microorganisms of humans and rodents. However, Western diets gained more attentions due to the changing composition of gut microbiota. Data from food sciences indicated that Western diets are rich in MRPs. Up to now, analyzing data on the actual impact of dietary MRPs on intestinal microbiota was scarce. While recent studies have provided robust evidence on the influence MRPs on gut bacteria *in vitro* studies, there are still a lot of gap in the current knowledge related to the effect dietary MRPs on gut microbiota *in vivo* studies. In this project, we assessed the impact of different MRPs (CML, bread melanoidins, and malt melanoidins) on the murine gut microbiota through three studies.

We concluded that the composition of gut microbiota of CML group was similar to the composition of gastrointestinal microbiota control group. Additionally, CML inhibited dysbiosis gut microbiota in DSS not TNBS models. This project showed that bread melanoidins have no bifidogenic effect, while malt melanoidins have bifidogenic effect. Both bread and malt melanoidins have no impact to increase *Lactobacillus*. Melanoidins appeared to have a prebiotic-like impact for the malts and inhibition of potentially pathogenic Proteobacteria for bread crust. Thus, melanoidins structure are extremely variable from one food to another, therefore additional studies will be needed to better assess their potential beneficial properties on gut health. Finally, we found that CML and melanoidins have no negative effects on the gut microbiota.

APPENDIX:

IACUC approval for mice study


To: Franck Carbonero
FR: Craig Coon
Date: November 28th, 2016
Subject: IACUC Approval
Expiration Date: November 24th, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED your protocol # 17033 *Impact of varying amounts of dietary Maillard products on healthy mice gut microbiota and associated biomarkers*.

In granting its approval, the IACUC has approved only the information provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond November 24th, 2017 you can submit a modification to extend project up to 3 years, or submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The following individuals are approved to work on this study: Franck Carbonero, and Nesreen ALjahdali. Please submit personnel additions to this protocol via the modification form prior to their start of work.

The IACUC appreciates your cooperation in complying with University and Federal guidelines involving animal subjects.

CNC/aem