UNIVERSITE DE LIEGE
FACULTE DE MEDECINE VETERINAIRE
DEPARTEMENT DES SCIENCES FONCTIONNELLES,
SERVICE DE PHARMACOLOGIE, PHARMACOTHERAPIE ET TOXICOLOGIE

LE RÔLE DU MICROBIOTE LORS D'INTOXICATION À L'HYPOGLYCINE A : ACTEUR OU TÉMOIN ?
Étude préliminaire

Anne-Christine FRANCOIS

MEMOIRE PRESENTE EN VUE DE L'OBTENTION DU GRADE DE DOCTEUR EN SCIENCES VETERINAIRES

ANNEE ACADEMIQUE 2018-2019

// REMERCIEMENTS

La réalisation de ce mémoire a été possible grâce au concours de plusieurs personnes à qui je voudrais témoigner toute ma gratitude.

Je voudrais tout d'abord adresser toute ma reconnaissance aux promoteur et co-promoteur, le Docteur D. VOTION et le Professeur P. GUSTIN , pour leur patience, leur disponibilité et surtout leurs judicieux conseils, qui ont contribué à alimenter ma réflexion.

Je tiens à remercier spécialement V. DELCENSERIE, qui m'a accueillie chaleureusement dans son service et qui m'a guidée dans ce mémoire, ainsi que C. DOUNY et B. TAMINIAU, qui m'ont permis d'obtenir une partie des résultats présentés dans ce travail grâce à leur inestimable expertise.

Je voudrais exprimer ma reconnaissance envers les amis et collègues qui m'ont apporté leur soutien moral et intellectuel tout au long de ma démarche : C. CAMBIER, C. KRUSE, S. LEBRUN, B. RENAUD, M. WEBER et T. ALLEN

Un grand merci à C. ANTOINE pour nos nombreux partages et notre entraide sur l'exploitation des résultats obtenus.

Enfin, je tiens à témoigner toute ma gratitude à ceux qui partagent mon quotidien pour leur confiance et leur soutien inestimable.

« La reconnaissance silencieuse ne sert à personne. »
Gladys Bronwyn Stern

LISTE DES ABREVIATIONS

HGA	Hypoglycin A
MCPG	α-(methylenecyclopropyl)glycine
JVS	Jamaican vomiting sickness
ATD	Acute toxic dose
MTD	Maximum tolerated dose
MCPA	Methylenecyclopropylacetyl
MCPF	Methylenecyclopropylformyl
MCPA-CoA	Methylenecyclopropylacetyl-CoA
MCPF-CoA	Methylenecyclopropylformyl-CoA
BCAD	Branched-chain-oxo-acid dehydrogenase
MCPA-Glycine	Methylenecyclopropylacetyl-glycine
MCPA-Carnitine	Metylenecyclopropylactetyl-carnitine
SHIME®	Simulator of the Human Intestinal Microbial Ecosystem®
N_2	Azote
SCFA	Short chain fatty acid
HTPLC	High-performance thin-layer chromatography
SD	Stomach/duodenum
JI	Jejunum/ileon
AC	Ascending colon
TC	Transverse colon
DC	Descending colon

TABLE DES MATIERES

RÉSUMÉ (FR) 1

MOTS CLES 1

TITLE 2

RESUME (EN) 2

KEYWORDS 2

INTRODUCTION 3

1. THE INTOXICATIONS WITH HYPOGLYCIN A 3

1.1. ATYPICAL MYOPATHY 3

1.2. ENCEPHALOPATHY IN HUMAN 4

1.3. JAMAICAN VOMITING SICKNESS IN HUMAN 4

2. THE TOXICOLOGICAL DOSES FOR HYPOGLYCIN A 5

2.1. MEDIAN LETHALITY DOSE OF HYPOGLYCIN A 5

2.2. ACUTE TOXIC DOSE OF HYPOGLYCIN A 6

2.3. MAXIMUM TOLERATED DOSE OF HYPOGLYCIN A 6

3. THE METABOLISM OF AMINO ACIDS AND HYPOGLYCIN A 6

3.1. METABOLISM OF AMINO ACIDS IN GENERAL 6

3.2. METABOLISM OF HYPOGLYCIN A 7

4. THE OBJECTIVE OF THE STUDY 9

MATERIALS AND METHODS 10

2.3. INTOXICATION WITH HYPOGLYCIN A 11

2.4. PRODUCTION OF SHORT CHAIN FATTY ACIDS 11

3. DETERMINATION OF STABILITY OF HYPOGLYCIN A IN THE NUTRITIONAL MEDIUM AND IN THE PANCREATIC JUICE 12

3.1. MATERIAL PREPARATION 12

3.2. SAMPLING METHOD 12

3.3. METHOD OF ANALYSIS 12

2. MODIFICATION OF MICROBIOTA FOLLOWING HGA INTOXICATION 13

2.1. SAMPLING METHOD 13

2.2. DIVERSITY ALPHA ANALYSIS 13

2.3 MICROBIOTA ANALYSIS 14

2.4 SHORT CHAIN FATTY ACIDS ANALYSIS 14

RESULTS 15

1. DETERMINATION OF STABILITY OF HYPOGLYCIN A IN THE NUTRITIONAL MEDIUM AND IN THE PANCREATIC JUICE 15

2. MODIFICATION OF MICROBIOTA FOLLOWING INTOXICATION WITH HYPOGLYCIN A 15

2.1. DIVERSITY ALPHA ANALYSIS 15

2.2. MICROBIOTA ANALYSIS 16

2.3. SHORT CHAIN FATTY ACIDS ANALYSIS 18

DISCUSSION 20

CONCLUSION 24

BIBLIOGRAPHY 25

RÉSUMÉ (FR)

INTRODUCTION : L’hypoglycine A est une toxine environnementale responsable d’intoxications sévères chez certains mammifères. Possédant la structure d’un acide aminé branché ,elle est transformée en un métabolite toxique : le méthylènecyclopropylacétyl-CoA. Cette métabolisation est réalisée grâce à un système enzymatique présent dans la majorité des cellules eukaryotes.
Le microbiote intestinal est capable de métaboliser les acides aminés branchés grâce à un système enzymatique similaire à celui utilisé pour métaboliser l’hypoglycine A. La question du rôle du microbiote lors d’intoxication à l’hypoglycine A se pose. Premièrement, la toxine est-elle capable de modifier le microbiote ? Est-ce que cette modification agit comme un acteur dans le processus pathophysiologique des différentes espèces cibles ? Et deuxièmement, cette toxine est-elle métabolisée par le microbiote ? Est-ce que ce métabolisme peut expliquer la sensibilité individuelle face à cette intoxication ? Ce mémoire propose une étude préliminaire visant à observer les changements du microbiote lors d’intoxication à cette toxine.

MATERIEL ET METHODES : Grâce à un simulateur de l’écosystème microbien intestinal humain, une intoxication à l’hypoglycine A a été reproduite . Les modifications du microbiote, la concentration de certains acides gras volatils produits par le microbiote sont observées ainsi que la stabilité de la toxine dans ce simulateur.

RESULTATS : Des modifications du microbiote sont observées comme la disparition des *Lachnospiraceae*, l’apparition des *Enterobacteriaceae* et l’installation d’une dysbiose observée, entre autre, par une diminution générale de la production des acides gras volatils. La concentration de la toxine semble instable dans le système. D’autres études seront nécessaires afin de confirmer ces observations.

MOTS CLES

Intoxication environnementale, Hypoglycine A, Microbiote, Méthylènecyclopropylacétyl-CoA

TITLE

The role of Microbiota in hypoglycin A intoxication: actor or witness?
Preliminary study

RESUME (EN)

INTRODUCTION: Hypoglycin A is an environmental toxin that causes severe intoxication in some mammals. Its chemical structure is that of a branched amino acid. It is metabolized in a toxic metabolite: the methylenecyclopropylacetyl-CoA. This metabolization is performed by an enzymatic system, which is present in various eukaryotic cells.
The intestinal microbiota is able to metabolize branched amino acids through an enzymatic system similar to that used to metabolize hypoglycin A in mammals. The question of the role of the microbiota in hypoglycin A poisoning arises: first, does the HGA modify the microbiota? If so, can this dysbiosis act as an actor in the pathophysiological process involved in the different target species? Secondly, is the toxin metabolized by the microbiota? If so, can this metabolism contribute to the highly variable sensitivity between species and individuals? The purpose of this preliminary study is to observe changes in the microbiota during hypoglycin A intoxication.

MATERIALS AND METHODS: Using a simulator of the human intestinal microbial ecosystem, hypoglycin A intoxication was reproduced. The microbiota changes and the production of short chain fatty acids are observed. The stability of the toxin inside the simulator is evaluated.

RESULTS: Changes in microbiota are observed after the intoxication with hypoglycin A: the *Lachnospiraceae* disappear, the *Enterobacteriaceae* appear. A dysbiosis is observed with the general decrease of short chain fatty acids. The toxin wasn't stable in the simulator. Further studies are needed to confirm these observations.

KEYWORDS

Environnemental intoxication, Hypoglycin A, Microbiota, Methylenecyclopropylacétyl-CoA

INTRODUCTION

Some trees of the *Sapindaceae* and *Hippocastanaceae* families contain toxins that may be responsible for environmental intoxication. For the time being, the identified toxins are (Fowden and Pratt, 1973): the β-(methylenecyclopropyl)alanine called hypoglycin A (HGA), the γ-glutamyl peptide of HGA called hypoglycin B, the α-(methylenecyclopropyl)glycin (MCPG) and the γ-glutamyl peptide of MCPG.
The *Sapindaceae* family include trees that produce edible fruits such as ackee, lytchee, longan or mamoncillo fruits. Studies of the chemical component in these fruits showed that they contain HGA and MCPG (Fowden and Pratt, 1973; Gray and Fowden, 1962; Hassal and Reyle, 1954; Isenberg et al., 2017, 2015; Melde et al., 1989; Sander et al., 2017).

Depending (1) on the proportion of each toxins present in the seeds or arils of the tree incriminated and (2) on the species involved, can generate very different disease:

- The atypical myopathy in horses and in Père David's deer following ingestion of seeds or seedlings from some tree of *Acer* species (Bunert et al., 2018; Valberg et al., 2013; Votion et al., 2014).
- The Jamaican Vomiting Sickness (JVS) (Hassal and Reyle, 1954; Joskow et al., 2006) and encephalopathies (Das and John, 2017; Mathew et al., 2017; Shrivastava et al., 2017) in human. These disease are due to ingestion of, respectively, ackee fruit from *Blighia Sapida* (Von Holt et al., 1964) and lytchee from *Litchi Chinensis* (Gray and Fowden, 1962; Melde et al., 1991), respectively.

Most of the scientific literature focused on the HGA toxin because it was identified in JVS intoxication (Sherratt, 1986) and the first one identified as the cause of atypical myopathy (Votion, 2018). Currently, the implication of other toxins in JVS or atypical myopathy are less studied.

1. THE INTOXICATIONS WITH HYPOGLYCIN A

1.1. ATYPICAL MYOPATHY

In horses, atypical myopathy is caused by HGA ingestion contained in seeds and seedlings of *Acer Pseudoplatanus* in Europe (Votion et al., 2014) and *Acer negundo* in the United States of America (US) (Valberg et al., 2013). The main clinical signs are weakness, recumbency, myoglobinuria, full bladder, stiffness, depression, muscle tremors or fasciculation, reluctance to move, sweating and congested membranes (Van Galen et al., 2012; Votion et al., 2007). Among risk factors for atypical myopathy, it has been proven that young horse and thin horses are more likely to develop this disease (van Galen et al., 2012). Recently, it has been showed that MCPG is also involved in the development of these diseases (Bochnia et al., 2019; Isenberg et al., 2017; Sanford et al., 2018).

Polygastric animals can develop similar symptoms to those observed in monogastric herbivore animals following ingestion of HGA from seeds or seedlings of *Acer* Species (Bunert et al., 2018). However, up to know, intoxication of ruminants has only been confirmed in animals kept in zoo, in enclosures characterized by a high toxic pressure (*i.e.* large number of trees).

1.2. ENCEPHALOPATHY IN HUMAN

Outbreaks of unexplained neurological illness with high mortality rate occurred in different countries in Asia (India, Bangladesh and Vietnam). A link between the development of this disease and lychee from *Litchi Chinensis* consumption was established (Isenberg et al., 2017; Mathew et al., 2017; Shrivastava et al., 2017; Zhang and Fontaine, 2017). Lychee are widely cultivated in China or in the Muzaffarpur region in India where the disease was encountered (Shrivastava et al., 2017; Zhang and Fontaine, 2017). Patients intoxicated following lychees ingestion presented convulsions or unconsciousness and hypoglycemia. Children were mainly involved : for example, children between 1 to 9 years represented 77% of cases in China (Isenberg et al., 2017; Shrivastava et al., 2017; Zhang and Fontaine, 2017).
Lychee contains more MCPG (44.9 µg/g to 220.0 µg/g) than HGA (12.4 µg/g to 152.0 µg/g of HGA) (Gray and Fowden, 1962; Isenberg et al., 2017; Shrivastava et al., 2017). This might explain the difference in clinical signs between the ingestion of lychee or ackee, the latter containing more HGA than MCPG.

1.3. JAMAICAN VOMITING SICKNESS IN HUMAN

The JVS is a disease caused by ingestion of ackee fruit, *Blighia Sapida*. The fruit is native to West Africa and is now cultivated in other regions. Ackee and canned ackee are staple food in Jamaica and a major export product in several subtropical countries (Sander et al., 2017).
Two fatal cases of JVS were explored in 1976. The authors concluded that this disease was linked with HGA in ackee fruit (Tanaka et al., 1976). Since then numbers of clinical cases have confirmed this conclusion including in other countries (Joskow et al., 2006).
In unripe fruit, the HGA level is elevated at concentrations of 1000 ppm. In ripe fruit, this level in only around or less than 100 ppm (Brown et al., 1991). Thus, it was concluded that clinical cases are due to the ingestion of unripe fruit. In the US, importation of ackee fruit is regulated by the Food and Drug Administration : only canned ackee fruit containing less than 100 mg of HGA/kg are allowed (Blake et al., 2004; Isenberg et al., 2015).

The clinical signs of JVS include nausea, vomiting, drowsiness and severe hypoglycemia. Clinical signs occur within 6-48 hour after ingestion of the fruit. In severe cases, coma and death can occur within 12 hours (Blake et al., 2004; Bressler et al., 1969; Brown et al., 1991). A profound hypoglycemia is noted

with glucose level as low as 3 mg/100 ml accompanied by depletion of glycogen store in the liver (Bressler et al., 1969).
The nutritional state seems to be an important trigger to the disease: indeed, patients with this disease are mostly malnourished or with vitamin deficiency. There is a slightly higher incidence of JVS in woman than in men (Bressler et al., 1969). Children between the aged of 2 and 5 are the most affected followed by children aged of 5 to 10 years (Bressler et al., 1969; Joskow et al., 2006; Sander et al., 2017).

In a study assessing the dietary exposure to HGA, Blake at al. (2004), determined the average HGA intake (µg/day/kg body weight) and the maximum HGA intake (µg/day/kg body weight) for Jamaican children (0-12 years), adolescents (13-19 years), adults (20-59 years) and the elderly (>60 years). These values are found in Table I. Interestingly, to the author's knowledge, no correlation was found between the ackee's quantity ingested and the toxics effects observed in human. This affirmation is probably due to the variability in HGA's quantities in each ackee that varied from 1.21 to 89.28 µg HGA/g ackee (Blake et al., 2004).

In order to advance toxicological evaluation around HGA, an adult male volunteer ate 1g ackee per kg of body weight with 99.2 µg HGA/kg of fruits in a first step of experiment and 5g lychee per kg of body weight with 1.3µg HGA/kg of fruits in a second experiment. The observation was that even a small quantity of ackee or lychee, that did not induce clinical symptoms, may be sufficient to detect (1) HGA, MCPG and their metabolites in serum or urine and (2) modification of acyl conjugates in the blood despite the lack of clinical symptoms. The presence of HGA or MCPG in blood or the presence of their metabolites in urine were only a sign of ingestion of the toxin and not the sign of possible acute illness (Sander et al., 2017).

2. THE TOXICOLOGICAL DOSES FOR HYPOGLYCIN A

2.1. MEDIAN LETHALITY DOSE OF HYPOGLYCIN A

In toxicology, the median lethality dose or LD50 is defined as the dose required to kill half of the population at a defined concentration of the toxic. In 1958, Feng et al, determined the LD50 of HGA on fed and on fasted rats. Grading doses of HGA were administered orally or intraperitoneally and the mortality was observed. The LD50 values were respectively 98 and 97 mg/kg of aqueous HGA extract (Feng and Patrick, 1958). No significantly difference were noted between fed or fasted rats, but it was observed that clinical signs following the administration of HGA were more important in fasted rats. This observation can be related to the fact that the majority of people developing JVS had an history of malnutrition (Chen et al., 1956; Feng and Patrick, 1958).

TABLE I

DIETARY INTAKE OF HYPOGLYCIN A BY JAMAICAN CONSUMERS CLASSIFIED BY AGE GROUP

	Average HGA intake (µg/day/kg body weight)	Maximum HGA intake (µg/day/kg body weight)
Children	8.18 +/- 6.04	115.2
Adolescent	7.41 +/- 3.52	79.30
Adult	6.55 +/- 3.12	69.90
Elderly	6.58 +/- 4.06	66.95

Average hypoglycin A intake (µg/day/kg body weight) and maximum hypoglycin A intake (µg/day/kg body weight) for Jamaican children (0-12 years), adolescent (13-19 years), adult (20-59 years) and elderly (>60 years).

In other studies, the LD50 for HGA was tested on different rodent species. In rats, the LD50 was 90 to 100mg/kg body weight in rats whereas mice survived with 160 mg HGA/kg. This value differs between species probably due to a variation in metabolism. Rabbits and monkeys were more sensitive: rabbits died after 10 to 20 mg HGA/kg intravenous and monkeys died after 20 to 40 mg HGA/kg intravenous within 5 days (Bressler et al., 1969; Chen et al., 1956; Hassal and Reyle, 1954).
The administration of a lethal dose of HGA to rats, rabbits and guinea-pigs was followed by a severe hypoglycemia in blood (< 30mg glucose/100ml blood) just before the death. The effects of one lethal dose HGA (250 mg/kg intramuscularly) induced a depletion in glycogen stores especially in liver and heart (Feng and Patrick, 1958).

2.2. ACUTE TOXIC DOSE OF HYPOGLYCIN A

Blake et al. (2006), fed rats with different concentrations of ackee fruit to determine the dose response in laboratory rats. The dose that caused impairment of movement, arched backs, raised hairs, 10% loss in body weight and blackening of feces in rats was defined as the acute toxic dose (ATD). In the study, this ADT was 231.19 +/- 62.55 mg HGA/kg of body weight for male and 215.99 +/- 63.33 mg HGA/kg of body weight for female. The matrix of the fruit probably induced a reduced absorption of hypoglycin in the gut of rats which might explain the difference with the LD50 in rats (Blake et al., 2006).

2.3. MAXIMUM TOLERATED DOSE OF HYPOGLYCIN A

The maximum tolerated dose (MTD) was the largest dose that does not induce overt toxicity (appreciable organ dysfunction, reduction in life span or 10% or greater retardation of body weight gain in growing animals compared with control animals). The MTD of HGA is 1.50 +/- 0.07 mg HGA/kg/day (Blake et al., 2006). By extrapolation, this dose has been assumed to be 26.5 mg HGA per horse (around 500 kg body weight) (Valberg et al., 2013).

It is worth noting that HGA or MCPG are not toxic agents as such (Von Holt et al., 1964). The toxic agents are their metabolites once linked with CoA, the methylenecyclopropylacetyl-CoA (MCPA-CoA) and the methylenecyclopropylformyl-CoA (MCPF-CoA), respectively (Melde et al., 1991; Tanaka, 1972). A better understanding of their metabolization and their fate in the gastrointestinal tract would help to find strategies to prevent these environmental diseases.

3. THE METABOLISM OF AMINO ACIDS AND HYPOGLYCIN A

3.1. METABOLISM OF AMINO ACIDS IN GENERAL

The HGA is an unusual amino acid as emphasized by its other name methylenecyclopropylalanine (Von Holt et al., 1964). Amino acids are mainly absorbed in small intestine to the portal bloodstream *via* two mechanisms: a Na^+-independent (facilitated transport) and a Na^+-dependent (secondary active transport) system. Theses transporters are located in the brush border and baso-lateral membranes of the enterocytes (Davila et al., 2013; Mailliard et al., 1995; Matthews, 1971). An important part of amino acids is used by the enterocytes (1) for local usage (amino acids permit the renewing of cells in the epithelial layer and the nutrient absorption) and (2) for production of metabolites. So the unmetabolized and *de novo* produced amino acids end up in the portal blood stream to the liver where they are metabolized or released in the peripheral circulation (Davila et al., 2013).
In the gut lumen, alimentary and endogenous content with intestinal microbiota are found. In humans and mono-gastric animals, bacteria reach a number of 10^4 cells per gram of digesta in the stomach to 10^{11} cells per gram of digesta in the large intestine (Dai et al., 2011; Davila et al., 2013). It is known that 30-50% of the dietary essential amino acids is not available for extra-intestinal tissues and less than 20% of the amino acid utilized by the small intestine is recovered in mucosal protein. The conclusion is that a part of the extracted amino acid is catabolized by the microbiota (Dai et al., 2012, 2010; Neis et al., 2015; Stoll et al., 1997). It is nowadays recognized that microbiota can use amino acids for their growth or to synthesize some amino acid (Dai et al., 2011, 2010; Davila et al., 2013).

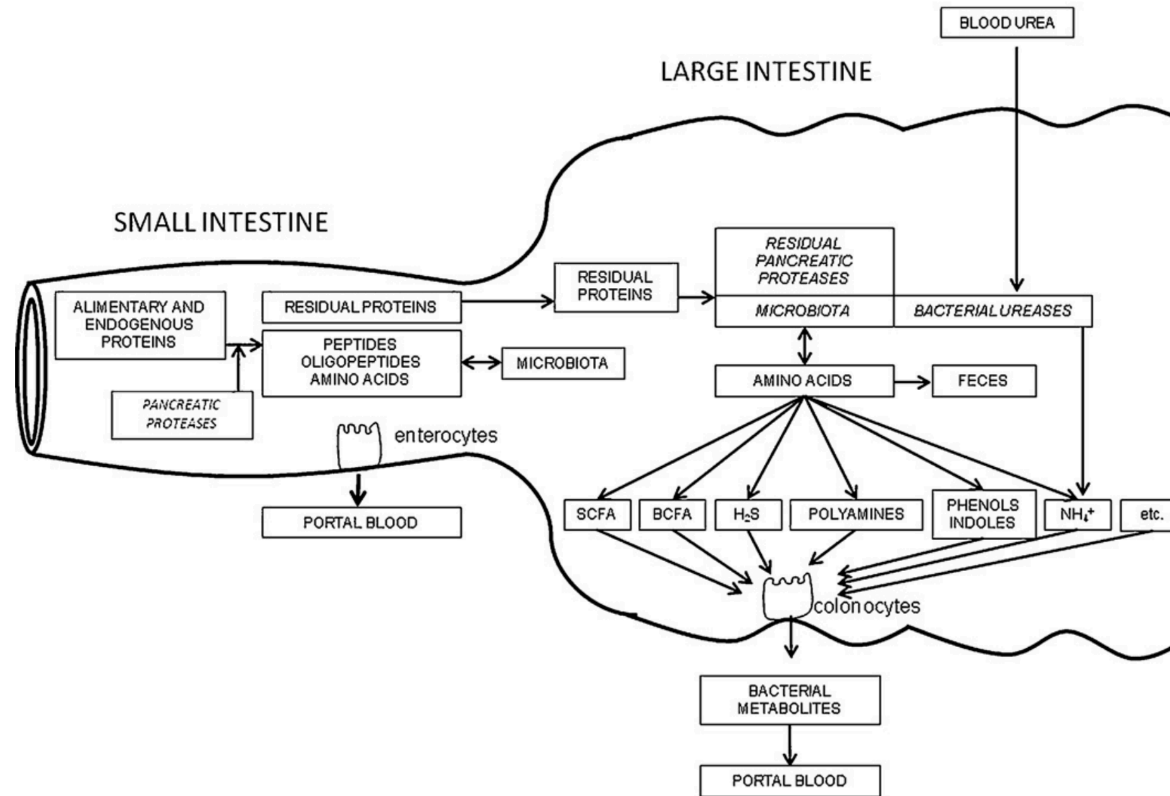
Unmetabolized residual proteins in the small intestinal lumen reach the large intestine. These proteins are subject to the action of residual pancreatic proteases and microbiota, which means that they are cleaved into amino acids. These amino acids are transformed by the microbiota into a mixture of metabolic end products: short-chain fatty acids, branched chain fatty acids, hydrogen sulfide, polyamines, phenols, ammonium…Some of these products are transported inside the colonocytes in order to reach the portal blood (Figure 1) (Davila et al., 2013).

3.2. METABOLISM OF HYPOGLYCIN A

It has been shown that HGA is metabolized in the liver (Von Holt et al., 1964). The first reversible step is a transamination in the cytosol which leads to the methylenecyclopropylpyruvic-acid. A second irreversible step of decarboxylation inside the mitochondria results in the toxic metabolite, MCPA-CoA (Billington and Sherratt, 1981; Melde et al., 1991; Tanaka, 1972). This step is the key step because of its irreversibility. Two enzymes are involved in these two steps : the branched-chain-amino-acid-aminotransferase and the branched-chain-oxo-acid dehydrogenase (BCAD) (Figure 2) (Melde et al., 1991).

MCPA-CoA inactivates acyl-CoA dehydrogenases (including butyryl-CoA and isovaleryl-CoA dehydrogenases) by covalent modification of the flavin prosthetic group. These acyl-CoA

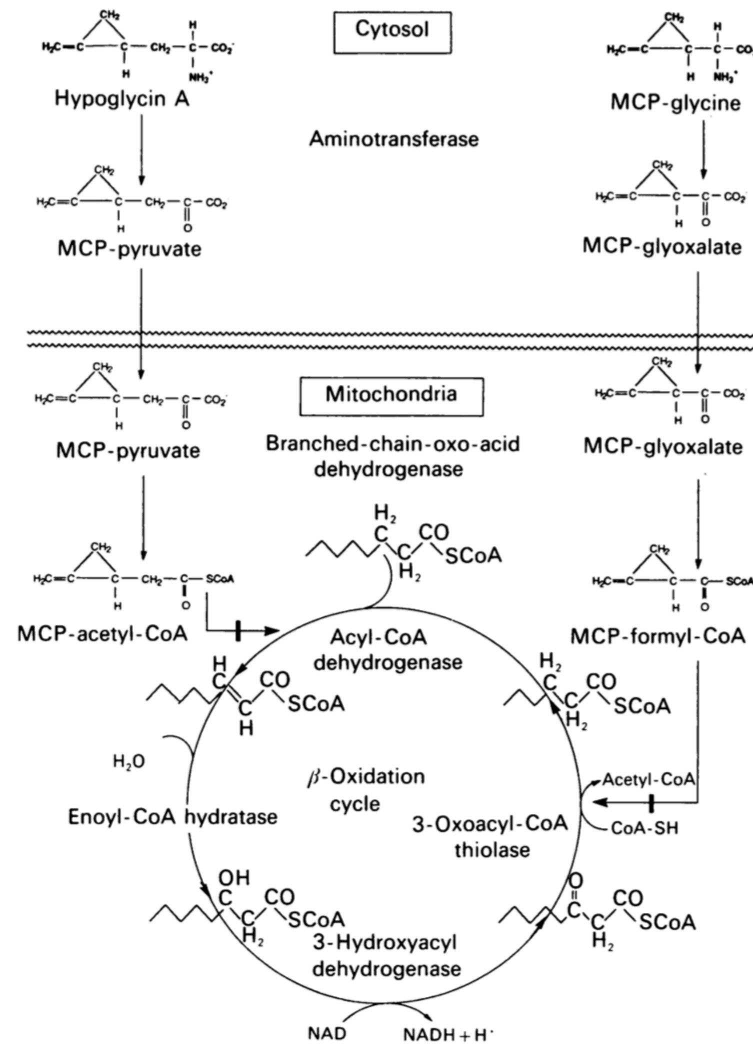
FIGURE 1



SCHEMATIC VIEW OF THE OVERALL METABOLISM OF LUMINAL PROTEINS IN THE SMALL AND LARGE INTESTINE (DAVILA ET AL., 2013)

Short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), hydrogen sulfide (H_2S), ammonium (NH^{4+})

FIGURE 2



CONVERSION OF HYPOGLYCIN A INTO ACTIVE METABOLITE AND THE SITE OF INHIBITION IN β-OXIDATION OF FATTY ACID (KLAUS MELDE ET AL., 1991B)

dehydrogenases are involved in the first step of fatty acid β-oxidation and in the degradation of branched chain amino acids like leucine, isoleucine and valine (Billington and Sherratt, 1981; Brooks and Audretsch, 1971; Melde et al., 1991; Sander et al., 2017; Tanaka et al., 1971; Wenz et al., 1981). The metabolization of MCPG follows the same two steps as HGA to become, *in fine*, MCPF-CoA. The toxic metabolite inhibits enoyl-CoA hydratase responsible of the second step of fatty acid β-oxidation (Melde et al., 1991). Both toxic metabolites act in synergy to impair fatty acid β-oxidation, since they inhibit different steps.

This acquired multiple Acyl-CoA dehydrogenase deficiency leads to an accumulation of different acyl-CoAs inside the mitochondria. Theses acyl-CoAs are converted into acylcarnitines. Therefore, blood concentrations of acylcarnitines are increased (Bressler et al., 1969; Mathew et al., 2017; Westermann et al., 2008).

MCPA and its glycine or carnitine conjugate are the end products of the metabolization of HGA to form the methylenecyclopropylacetyl-glycine (MCPA-Glycine) or metylenecyclopropylactetyl-carnitine (MCPA-Carnitine) in urine or blood (Billington and Sherratt, 1981; Isenberg et al., 2015; Sander et al., 2017). In rats, the amount of MCPA-Glycine excreted in urine represent 25 – 40% of the administered HGA (Tanaka, 1972). This major route of elimination via urinary excretion is the same for MCPG (Sander et al., 2017).

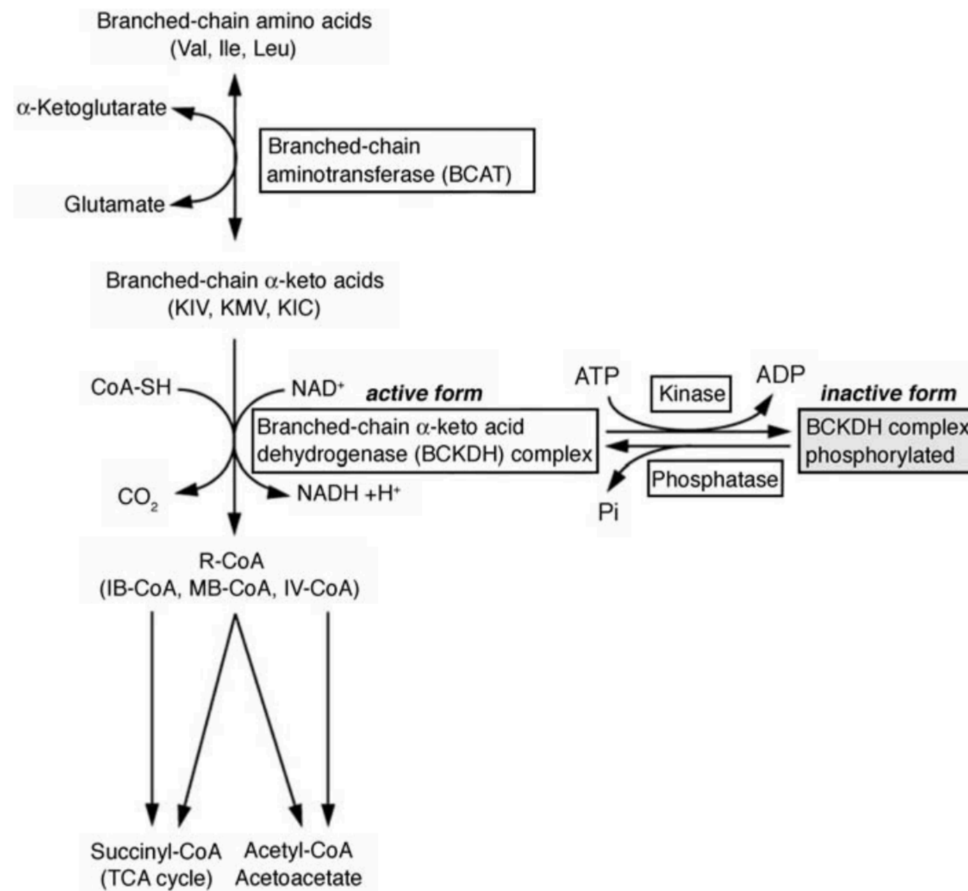
The second key enzyme, the BCAD, is present within the mitochondria and is therefore is present in all eukaryotic cells. This enzyme is part of an enzymatic complex that catalyzes an irreversible step in the catabolism of several amino acids as well as branched-chain amino acids (Yeaman, 1989).

Indeed, branched-chain amino acids are metabolized in human and in bacteria in a two-steps system. The initial step is a reversible transamination that results in the corresponding branched-chain α-ketoacid in the cytosol of the mitochondria by a branched-chain amino-acid aminotransferase. The second step is an irreversible oxidative decarboxylation by a branched-chain α-ketoacid dehydrogenase to lead to the branched-chain acyl-CoA intermediates. This enzyme is a complex located on the inner mitochondrial membrane (Figure 3) (Adeva-Andany et al., 2017; Brosnan and Brosnan, 2006; Harper et al., 1984; Massey et al., 1976).

In primates, this branched-chain α-ketoacid dehydrogenase is distributed as follow: 30% in liver, 2% in kidney and 60% in muscle (Khatra et al., 1977). These enzymes are present in some bacteria such as, for example, *Streptococcus faecalis, Proteus Vulgaris, Pseudomonas aeruginosa, Pseudomonas putida, Staphylococcus aureus, Bacillus subtilis* (Dickinson and Dawes, 2009; Massey et al., 1976; McCully et al., 2015; Singh et al., 2008).

As several bacteria are able to metabolize branched-chain amino acids, it is hypothesized that bacteria present in the microbiota could play a role during intoxication with HGA. In recent years, in vitro models

FIGURE 3



THE BRANCHED CHAIN AMINO-ACID CATABOLIC PATHWAY (BROSNAN AND BROSNAN, 2006)

have been developed to dynamically mimic microbial processes in the gastro-intestinal tract. For example, the SHIME®, the Simulator of the Human Intestinal Microbial Ecosystem, is a multicompartment dynamic simulator of the human gut (ProDigest) that allows the study of microbiota along the digestive tract. The main feature of the SHIME® is the possibility to differentiate the upper digestive tract (stomach, small intestine) and the lower digestive tract (ascending, transverse and descending colon) thanks to double-jacketed glass containers, which represent the different parts of digestive tract. This in vitro simulator allows quantification of short chain fatty acid (SCFAs) produced by the microbiota.

4. THE OBJECTIVE OF THE STUDY

The intestinal microbiota is able to absorb and metabolize branched amino acids through an enzymatic system similar to that used to metabolize HGA in mammals. The question of the role of the microbiota in HGA poisoning arises: first, does the HGA modify the microbiota? If so, can this dysbiosis act as an actor in the pathophysiological process involved in the different target species? Secondly, is the toxin metabolized by the microbiota? If so, can this metabolism contribute to the highly variable sensitivity between species and individuals?

The SHIME® is now recognized as a reference method to investigate the influence of external factors on the microbiota especially on microbiological and biochemical aspects and on the biotransformation activity of xenobiotics. The SHIME® being now validated for the human microbiota and owing the high sensitivity of children to HGA exposure (children with a mainly solid diet), this pilot study has been conducted on the microbiota of a 3-year-old child.

MATERIALS AND METHODS

1. DETERMINATION OF THE DOSE OF HYPOGLYCIN A

Taking into account the limit of quantification of our analytical method (Habyarimana et al., 2017) and the need to use a first experimental dosage suspected of causing toxicological effects, the daily dose injected to the SHIME® selected in this pilot study was 10 times the average food daily intake of a 3-year-old children weighing 13kg (8.18 +/- 6.04 µg/day/kg body weight (Blake et al., 2004)), and was calculated as follow:

((Average HGA intake x 10) x average weight of a 3-year-old child) x 6 days = HGA/week(µg)

((8.18 µg HGA x 10) x 13) x 6 = 6380.4 µg HGA/week

As the commercially available HGA (Toronto Research Chemicals Inc (TRC®, Toronto, Canada)) was provided as 1mg/tube, we chose to administer 6 mg in the feed for the challenge in order to avoid dissolution or picking error.

2. DESCRIPTION OF THE SIMULATOR OF THE HUMAN INTESTINAL MICROBIAL ECOSYSTEM®

2.1. GENERAL DESCRIPTION OF THE SHIME®

In this experiment, the SHIME® was divided in 5 compartments: stomach/duodenum (SD), jejunum/ileum (JI), ascending colon (AC), transverse colon (TC), descending colon (DC). The system was completely close and sealed. The temperature was maintained at 37°C thanks to a water bath that allows water circulation at a constant temperature in the system. Anaerobic conditions were kept by a nitrogen flow 10 minutes a day (flow rate: 2L/minute). The pH was maintained between 5.4-5.8 in AC, 6.0-6.3 in TC and 6.3-6.5 in DC. We constantly controlled the pH and adjusted it if needed by adding NaOH (0.5 M) or HCL (0.5 M). The volume of the three “colon-compartments” was kept to 150 mL for AC, 240 mL for TC and 180 mL for DC. Retention times vary from 24 to 72h according to the human group of interest (Van de Wiele et al., 2015).

A nutritional medium, the “feed”, was added three times a day to the gastric compartment and contained complex carbohydrates, proteins sources with mucins and mineral and vitamin complex. In the first study aiming the validation of the system, this medium consisted of (g/L): 1.0 arabinogalactan, 2.0 pectin, 1.0 xylan, 3.0 starch, 0.4 glucose, 3.0 yeast extract, 1.0 proteose peptone, 4.0. mucine, 0.5 cysteine (Molly et al., 1994; Possemiers et al., 2004). The pancreatic and bile liquid were added three times a day to the small intestine container. They contained a mix of oxgall, pancreatin and NaHCO3 (Possemiers et al., 2004). A peristaltic pump created the progression of the digestive content through the different part of the tract and magnetic stir bars allowed its homogenization in the containers. The

total volume represented 60 mL: 42 mL for the feed and 18 mL for the pancreatic juice. A new feed and a new pancreatic juice were prepared and connected to the SHIME® every week after sampling planned the same day (Figure 4).

2.2.INOCULATION WITH MICROBIOME

A fecal microbiome was inoculated in the SHIME®. A stabilization time is needed to allow to the fecal microbiota to be a colon region-specific microbiota under imposed conditions by the SHIME® (Venema and Van den Abbeele, 2013). A period of at least 15 days is needed to stabilize the production of short chain fatty acid (Van de Wiele et al., 2015).

In this study, an inoculum from a 3-year-old child was used after a specific preparation. This preparation consisted in (1) a 20% dilution in phosphate buffer (2) homogenization and (3) filtration with a laboratory blender (Interscience BagMixer® 400). A two-weeks stabilization period was performed to allow microbiota to develop and adapt specifically to each portion of the *in vitro* digestive tract.

2.3.INTOXICATION WITH HYPOGLYCIN A

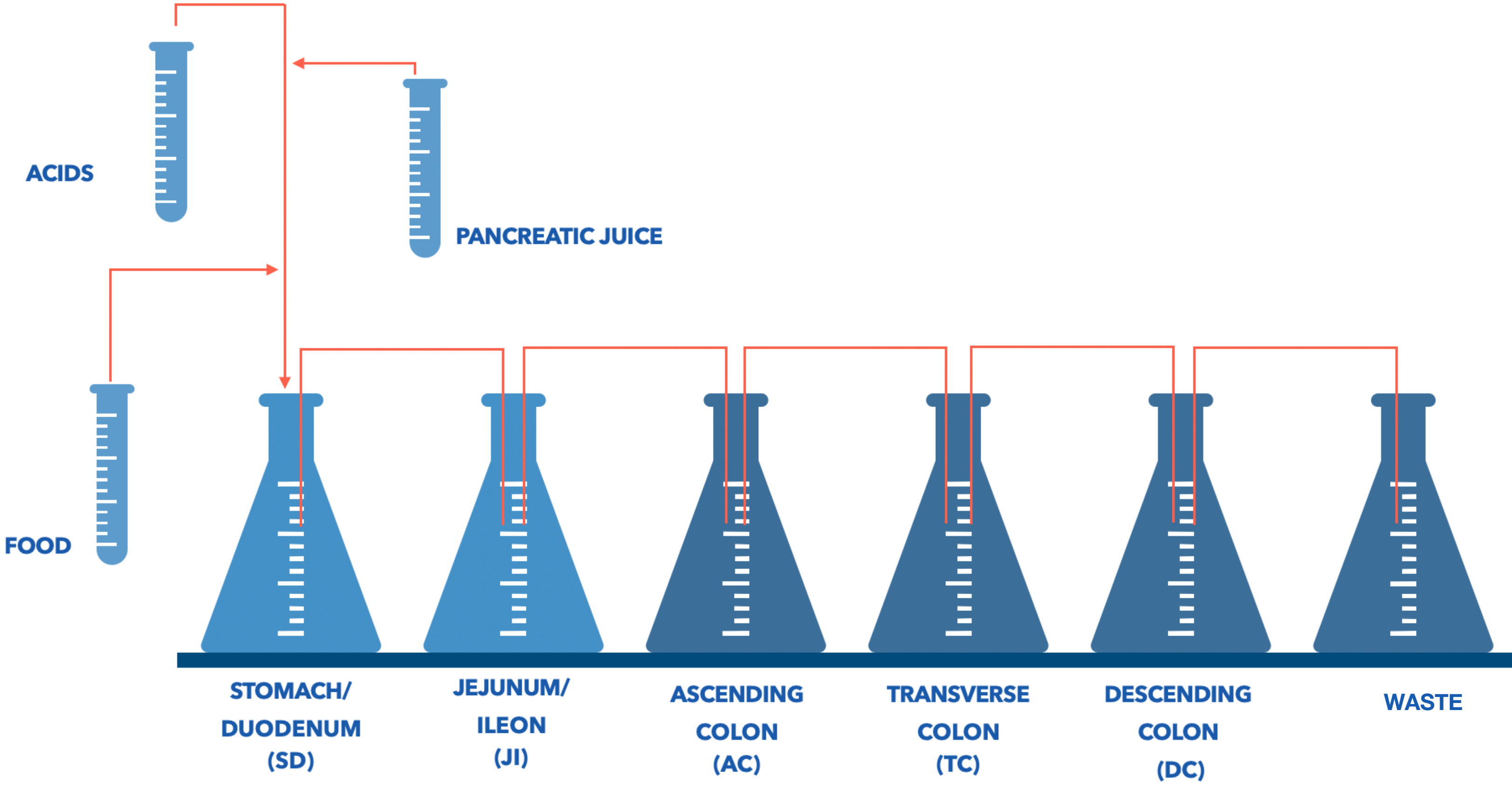
After this stabilization period, a 6-days challenge was realized by adding 6 mg of purified commercially obtained HGA (Toronto Research Chemicals Inc (TRC®, Toronto, Canada)) in the feed. During this challenge, samples were also taken 3 times/week in each colon compartment.

The day of introduction of HGA is called "J". The sample is taken before changing the feed and so before adding HGA. These samples called J represented the system at the end of the stabilization period and just before the intoxication. Two days later, other samples were taken and labelled "J+2". These samples were under the influence of HGA for 48 hours. The challenge stopped after taking the samples called "J+7" because of the introduction of a new feed without HGA. The samples called "J+9" are free from the influence of HGA for 48 hours. After this challenge, an additional week without HGA in the feed was performed.

2.4.PRODUCTION OF SHORT CHAIN FATTY ACIDS

This *in vitro* simulator allows quantification of short chain fatty acid (SCFAs) produced by the microbiota. They are fatty acid with a carbon number between 2 and 6 and are called as follow: C2 acetic, C3 propionic, C4 butyric, C5 valeric and C6 caproic acid. In animals, majors SCFAs are acetate, propionate and butyrate. In humans, these three main SCFAs are produced by microbiota degrading dietary fibers and resistant starches (Neis et al., 2015; Ohira et al., 2017).

FIGURE 4



SCHEMATIC REPRESENTATION OF SHIME®

3. DETERMINATION OF STABILITY OF HYPOGLYCIN A IN THE NUTRITIONAL MEDIUM AND IN THE PANCREATIC JUICE

3.1.MATERIAL PREPARATION

In order to verify the HGA's stability in the nutritional medium, 120 µg of HGA was added to 20 mL feed for one week. A magnetic stir bar was used to allow homogenization and samples were taken three times a day for one week.

A second step in the stability check of HGA in SHIME® was to verify its stability in nutritional medium added with pancreatic juice for 90 minutes. A volume of 4,3 mL of pancreatic juice was added to another preparation of 10 mL of feed, 60 µg of HGA was added to the solutions.

3.2.SAMPLING METHOD

Three samples of 1 mL of nutritional medium containing HGA were taken with a graduated transfer pipet of 3 ml within one week to reproduce the conditions that were used for the SHIME's run.

The other three 1 mL-samples of nutritional medium with pancreatic juice and HGA were taken with a graduated transfer pipet every 30 minutes for 90 minutes to reproduce the average time of contact between the two media in the system.

3.3.METHOD OF ANALYSIS

The 1 ml-samples were evaporated with an air flow at ambient temperature. Once completely dry, 50 µL of purified water were added (Système Simplicity® UV, Millipore S.A.S., France). The samples were placed is one ultrasound tank in order to suspend the dry material for 5 minutes and vortexed. The temperature was monitored so as not to exceed 50° Celsius. Indeed, the HGA is stable up to 50° Celsius (Gonzalez-Medina et al., 2018). The samples were centrifuged in order to sample the supernatant. This was done because the samples were too dense to allow a valid depot on the high-performance thin-layer chromatography (HTPLC) plate. The results would have been unreadable. An extraction was performed in order to limit the feed contamination in the samples.

From the 50 µL of the sample, 20 µL was taken. A volume of 100µL of methanol was added to the 20µL (total volume 120 µL) and placed for agitation during 24 hours in 50°C. A centrifugation was performed. A volume of 60 µL of supernatant was used for an evaporation step with air flow until the sample were totally dry at ambient temperature. Finally, 60 µL of purified water was added in order to eliminate the methanol. A 3 µL sample from the last extract was used as depot for the HTPLC method.

The various compounds present in the extract were separated by chromatography on silica plates (60Å F254, 20 × 10 cm or 10 × 10, Merck Darmstadt, Germany). HGA (purity> 95%) obtained via Toronto

Chemicals (Toronto, Canada) was used as a standard. The standard and the extracts were deposited in 4 mm wide band with a volume of 3 µL, using a Hamilton® 100 µl syringe mounted on a CAMAG Linomat 5® applicator. Each plate was allowed to dry at room temperature and under hood for 15 minutes before starting the migration. Elution was carried out with methanol / acetic acid / water (70/20/10, v / v / v). After the migration, the plate was post-chromatographically derivatized by three successive sprays of ninhydrin (0.2% solution in methanol) and then incubated for 5 minutes at 115 ° C. The plates were read at a frequency of 490 nm by a CAMAG® TLC scanner 3 combined with the WinCATS© 4.3 software (CAMAG, Muttenz, Switzerland).

2. MODIFICATION OF MICROBIOTA FOLLOWING HGA INTOXICATION

2.1.SAMPLING METHOD

The three colon-compartments were each one connected to a tubing linked to a 20 mL syringe. To take the sample, every syringe was fill in and fill out 10 times by the same operator to minimize operator effect and to homogenize content. A 15ml-sample was taken by compartment and distributed to 3 Falcon-tubes of 15 mL (4 mL for each tube) and 2 Eppendorf-tube of 1.5 mL three times a week. One Eppendorf-tube was centrifuged during 2 minutes at 13000 rpm. The supernatant was sampled and placed in another empty Eppendorf-tube and the bottom of the Eppendorf-tube was conserved as such. All the samples were placed in a -80°C freezer until additional analyzes. This sampling was performed 3 times/week. The day of the inoculation of the system, one 15 mL- sample is taken from TC and distributed into Falcon-tubes and Eppendorf-tubes as described above.

2.2.DIVERSITY ALPHA ANALYSIS

Alpha diversity is a measure of the diversity of a single sample. The use of diversity indices is necessary to calculate and quantify the diversity status. These indices estimate biological and ecological quality (Bandeira et al., 2013). Three indices are used for this analyze: (1) the Shannon index, (2) the Simpson's diversity index and (3) the Fisher index.

- The Shannon index correspond with the measurement of the physical entropy. If the abundance is based on one specie and the other species are rare, this Shannon index will be near to zero (Bandeira et al., 2013).

- The Simpson's Reciprocal diversity index is used to quantify the biodiversity and taking into account the number of species and their abundance in the sample. The value of this index can vary from 1 (only one specie) to a higher value (higher diversity) (Bandeira et al., 2013; "Simpsons Diversity Index," n.d.).

- The Fisher index describe the relation between the number of species and the number of individuals in those species (Fisher et al., 1943).

2.3 MICROBIOTA ANALYSIS

As described by Ngo et al., (2018), total bacterial DNA was extracted from the stool samples with the PSP Spin Stool DNA Plus Kit 00310 (Invitek), following the manufacturer's recommendations. PCR-amplification of the 16S rDNA V1–V3 hypervariable region and library preparation were performed with the following primers (with Illumina overhand adapters), forward (50-GAGAGTTTGATYMTGGCTCAG-30) and reverse (50-ACCGCGGCTGCTGGCAC-30). Each PCR product was purified with the Agencourt AMPure XP beads kit (Beckman Coulter; Pasadena, CA, USA) and submitted to a second PCR round for indexing, using the Nextera XT index primers 1 and 2. After purification, PCR products were quantified using the Quant-IT PicoGreen (ThermoFisher Scientific; Waltham, MA, USA) and diluted to 10 ng/µl[BT1] . A final quantification of each library was performed using the KAPA SYBR® FAST qPCR Kit (KapaBiosystems; Wilmington, MA, USA) before normalization, pooling and sequencing on a MiSeq sequencer using V3 reagents (Illumina; San Diego, CA, USA). Positive control using DNA from 20 defined bacterial species and a negative control (from the PCR step) were included in the sequencing run (Ngo et al., 2018).

The V1-V3 regions are the regions were the most reliable regions in the full-length 16S rDNA sequences (Zhang et al., 2018). The 16S amplicon sequencing is one of the major method to determine bacterial community (Allen et al., 2016).

2.4 SHORT CHAIN FATTY ACIDS ANALYSIS

SCFA concentration were analyzed by solid phase microextraction, gas chromatography and mass spectrometry according to Douny et al. (2019). With each series of samples, a calibration curve was prepared to be used for the quantification and two quality control samples were analyzed to assess the performances of the method.
Briefly, 25 µL of each sample (including quality control samples) were mixed with 40 µL of internal standard solution (2-methyl valeric acid, 0.2 mg/mL), 15 µL de H_2SO_4 0,9M (sulfuric acid) and 920 µL of culture medium (also mentioned as the feed). All samples were vortexed before analysis. SCFA were extracted with a DVB/CAR/PDMS SPME fiber (Supelco, Bellefonte, PA, USA), separated on a Focus GC gas chromatograph (Thermo Fisher Scientific) using a Supelcowax-10 column (30 m × 0.25 mm, 0.2 µm) (Supelco) and analyzed with an ion trap PolarisQ mass spectrometer (Thermo Fisher Scientific). The concentrations of each SCFA were calculated by Xcalibur 3.0.63 program. The values are in mg/L (Douny et al., 2019).

1. DETERMINATION OF STABILITY OF HYPOGLYCIN A IN THE NUTRITIONAL MEDIUM AND IN THE PANCREATIC JUICE

The results of the two experiments are expressed in microgram per milliliter. Concentrations of HGA in the nutritional medium and the pancreatic juice were measured for 90 minutes and are noted in (Table II). Concentrations of HGA in the nutritional medium were measured for one week are noted in (Table III). No major problem was encountered. A negative control, a positive control and different standards were used to assess the validity of the HTPLC. One sample, called “Feed + HGA "J+2", has no value: the reason is the lack of deposit on the chromatography plate.

In the nutritional medium and the pancreatic juice, the concentration of HGA is stable and ranges from 84.42 µg/mL to 81.47 µg/mL. However, in the second experiment, concentrations of HGA decreased during the week and varied from 107.44 µg/mL to 24.52 µg/mL. Peaks of HGA were present in each sample, even though a slightly different form from the standards used in the experiment was noticed (Figure 5, Figure 6). To explain this difference, another sample of feed without HGA was tested and a peak compatible with HGA was present (Figure 6).

2. MODIFICATION OF MICROBIOTA FOLLOWING INTOXICATION WITH HYPOGLYCIN A

2.1. DIVERSITY ALPHA ANALYSIS

These three indexes were calculated for each colon before and after the introduction of HGA in the SHIME®.

For the AC, before and after introduction of HGA:

- The Shannon index is of 0.11 and 0.38 respectively
- The Simpson’s Reciprocal diversity index is of 1.1 and 1.76 respectively
- The Fisher index is of 1.74 and 2.9 respectively

Inside the TC, before and after introduction of HGA:

- The Shannon index is of 1.11 and 0.77 respectively
- The Simpson’s Reciprocal diversity index is of 6.05 and 3.33 respectively
- The Fisher index is of 13.26 and 9.22 respectively

Finally, for the DC, before and after introduction of HGA:

- The Shannon index is of 1 and 0.41 respectively
- The Simpson’s Reciprocal diversity index is of 4.47 and 1.72 respectively

TABLE II

CONCENTRATIONS OF HGA IN THE NUTRITIONAL MEDIUM AND THE PANCREATIC JUICE

	HGA µg/mL
Feed +Pancreatic juice + HGA T0'	84.42
Feed +Pancreatic juice + HGA T30'	74.54
Feed +Pancreatic juice + HGA T60'	82.89
Feed +Pancreatic juice + HGA T90'	81.47
Upper limit (UL)	98.23
Lower limit (LL)	10.75

The evolution of the concentration of HGA in the nutritional medium and in the pancreatic juice for 90 minutes. Concentrations are expressed in microgram per milliliter.

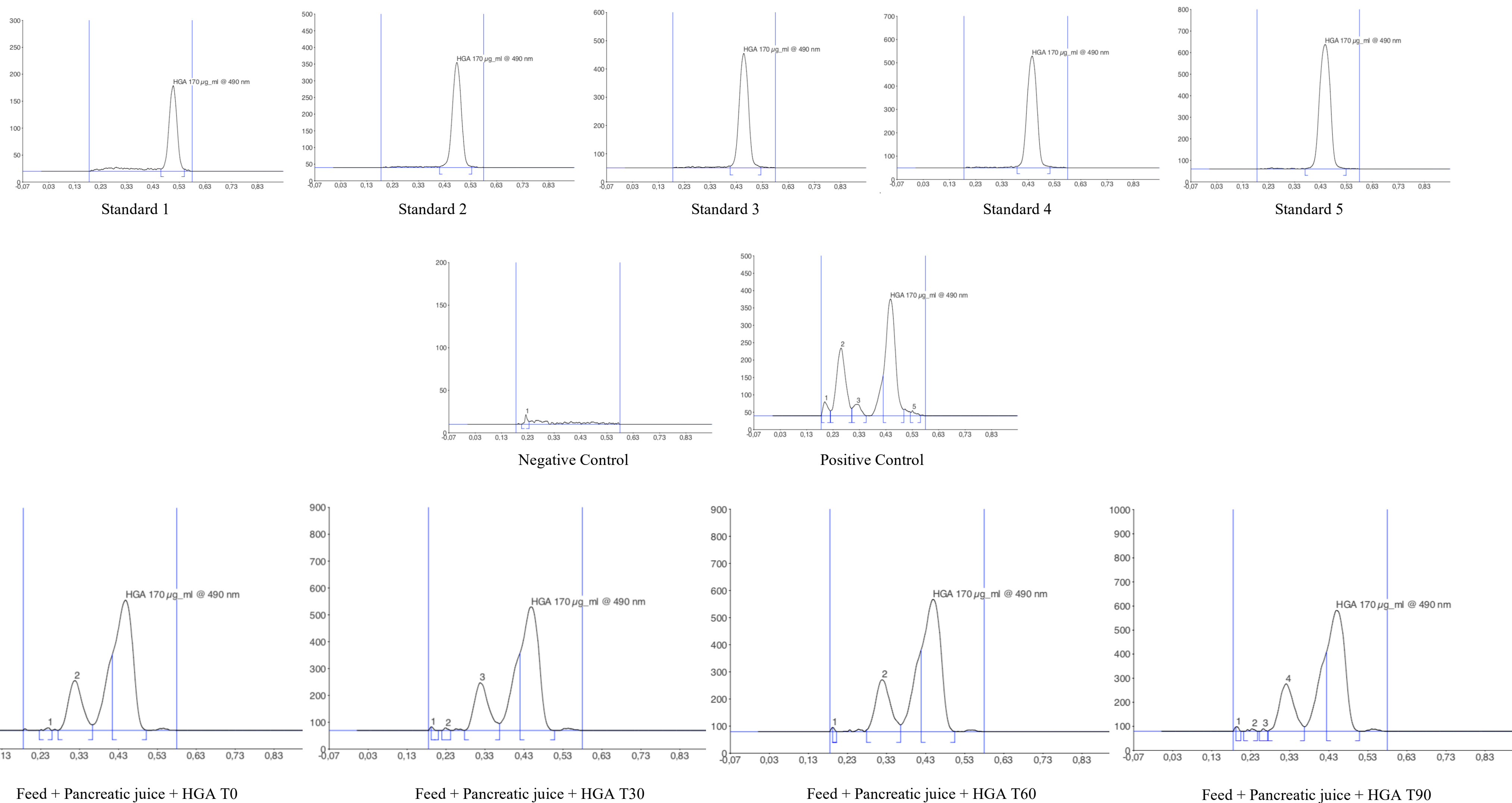
TABLE III

CONCENTRATIONS OF HGA IN THE NUTRITIONAL MEDIUM

	HGA µg/mL
Feed without HGA "J"	49.15
Feed + HGA "J"	107.44*
Feed + HGA "J+1"	117.37*
Feed + HGA "J+2"	/
Feed + HGA "J+3"	64.58*
Feed + HGA "J+4"	24.52*
Feed + HGA "J+7"	< LL
Upper limit (UL)	108.85
Lower limit (LL)	18.73

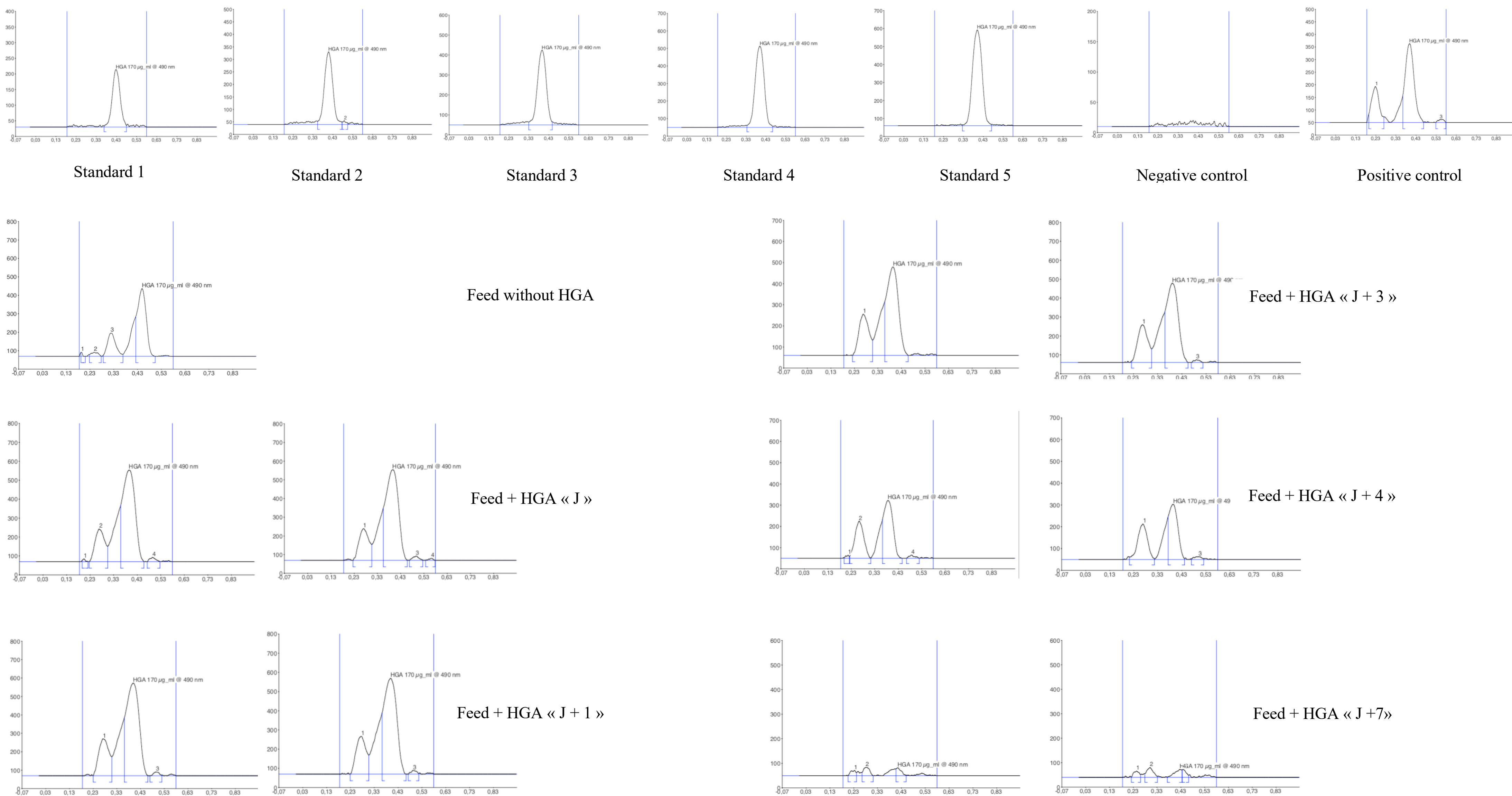
The evolution of the concentration of HGA in the nutritional medium for one week. Concentrations are expressed in microgram per milliliter. Results that were below the lower limit of detection are expressed with “<LL”. The values with * are the average between two values.

FIGURE 5



RESULTS OF HTPLC: PEAKS OF CONCENTRATION OF HGA (µg/mL) IN STANDARD, IN NEGATIVE AND POSITIVE CONTROLS AND IN FEED WITH PANCREATIC JUICE

FIGURE 6



RESULTS OF HTPLC: PEAKS OF CONCENTRATION OF HGA (µg/mL) IN STANDARD, IN NEGATIVE AND POSITIVE CONTROLS AND IN FEED

- The Fisher index is of 14.64 and 4.72 respectively

2.2.MICROBIOTA ANALYSIS

The results are expressed in relative abundance (percentage) and not in absolute abundance with the exact number of each bacteria. There are six bacterial phyla present during this SHIME run: *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Protebacteria* and *Verrucomicrobia* (Figure 7). However, three of them are the most represented and showed the most significant changes during the study: *Bacteroidetes, Firmicutes* and *Protebacteria* (Figure 7, Table IV). That's the reason why the not significant modifications of the three other phyla will be not discussed. The same method is applied for families inside the phylum and for genera inside the family: only bacterial families or genera with important changes are discussed. That's why, in bacterial families, the following families, the following families are the only one discussed: *Bacteroidaceae, Clostridiaceae_1, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, Enterobacteriaceae*. And, in bacterial genera, only the following genera are discussed: *Bacteroides, Clostridium butyricum, Lachnoclostridium, Megamonas Rupellensis, Megashaera, Veillonella Parvula, Enterobacteriaceaea, Escherichia-Shigella, Klebsiella pneumoniae, Pseudomonas aeruginosa*.

ASCENDING COLON

After the stabilization period, the microbiota is composed by 99.95% of *Firmicutes*. There is no evidence of change of *Firmicutes* phylum after the intoxication with HGA (Figure 7, Table IV). However, inside this phylum, two families show significant changes:

- The *Clostridiaceae* family (*Clostridia* class) appears in the end of the stabilization time with a relative abundance of 50.66% at "J-3" and 97.12% at "J". Its abundance decreases after the administration of HGA up to 0.12% (Figure 8, Table V). This family is mainly represented by *Clostridium butyricum* genus.
    - This genus follows the same evolution with a relative abundance of 47.81% at "J-3" and of 95.1% at the end of the stabilization period. *Clostridium butyricum* drops to 0.12% at the end of the challenge with HGA (Figure 9, Table VI).
- The *Veillonellaceae* family (*Negativicutes* class) is the main family during the stabilization period but decreases at the end of this period from 49.16% at "J-3" and 2.54% at "J". After the introduction of HGA, the *Veillonellaceae* family increase to 98.69% (Figure 8, Table V). Three main genera are involved in the changes observed for this family:
    - *Megamonas Rupellensis*: This genus presents a relative abundance over 90% during the stabilization period, which explains the relative abundance of the *Veillonellaceae* family. At the end of this stabilization, it drops during the days "J-3" and "J". After adding HGA, the relative abundance increases to 75.48% (Figure 9, Table VI).

- *Veillonella* Parvula presents an increasing relative abundance to 19.77% after the introduction of HGA (Figure 9, Table VI).
- *Megasphaera micronuciformis* presents an increasing relative abundance to 3.43% after the intoxication with HGA (Figure 9, Table VI).

This decrease in *Veillonellaceae* family is opposite to the increase of the *Clostridiaceae* family which could explains the constant relative abundance of *Firmicutes* phylum.

TRANSVERSE COLON

At the end of the stabilization period (Day "J"), the microbiota is mostly composed by *Firmicutes* (84.33%), *Bacteroidetes* (14.09%) and *Proteobacteria* (1.58%). After HGA intoxication (Day "J+7"), *Firmicutes* drop to 52.98%, *Bacteroidetes* drop to 8.31% and *Proteobacteria* increase to 38.68% Figure 7, Table IV).

Inside the major phyla *Firmicutes*, two bacterial families are mainly present:

- The *Veillonellaceae* family which represents 39.95% of the microbiota before the intoxication with HGA. With the addition of HGA, its relative abundance slightly increases. This modification is maintained until the end of the experiment (Figure 8, Table V).
  - The *Megamonas Rupellensis* genus is the major representant of this family. Its relative abundance follows the relative abundance of the family during the whole experiment in transverse colon (Figure 9, Table VI).
- The *Lachnospiraceae* family which represents 39.37% of the microbiota at the day "J". One of the major changes after the one-week intoxication with HGA concerns this family. Indeed, its relative abundance sharply drops to 2.21%. This variation is maintained during the experiment including after stopping the toxin's administration (Figure 8, Table V).
  - Inside this family, the major genus is *Lachnoclostridium.* Its relative abundance is close to that one of the family with a relative abundance equal to 33.74% at day "J" and to 1.52% after the contact with HGA (Figure 9, Table VI).

For the *Bacteroidetes* phyla, the main bacterial family is

- The *Bacteroidaceae* family with a relative abundance of 11.59% following the stabilization period and of 8.18% after the one-week challenge. The modification is maintained until the end of the experiment (Figure 8, Table V).
  - The relative abundance of the genus *Bacteroidetes* explains the changes observed on this family and in the phylum (Figure 9, Table VI).

Concerning the *Proteobacteria* phyla, the main family involved is

- The *Enterobacteriaceae* family which undergoes a severs increase after the intoxication with HGA. In fact, the relative abundance of this family goes from 0.065% to 36.24%. This is the second major change observed in transverse colon (Figure 8, Table V).

    - This increase is mainly due to the increase of *Citrobacter* (32%) and *Escherichia-Shigella* (1.56%) (Figure 9, Table VI).
- The *Pseudomonas aeruginosa* genus is also involved in the evolution of *Proteobacteria* phyla with a relative abundance of 2.29% after the intoxication with HGA (Figure 9, Table VI).

DESCENDING COLON

The microbiota in the DC looks like the microbiota in the TC after the stabilization period with 85,92% of *Firmicutes,* 12,62% of *Bacteroidetes* and 1.45% of *Proteobacteria*. The changes after intoxication with HGA are similar with a drop of *Firmicutes* to 2.83% and *Bacteroidetes* to 3.33% but with an increase of *Proteobacteria* to 93.81% (Figure 7, Table IV). The two major changes observed in TC are observed in DC:

- The decrease of *Lachnospiraceae* family from 30.46% to 17.81% (Figure 8, Table V).
    - The genus *Lachnoclostridium* is the most abundant in this family and therefore follows the same evolution with a drop from 51.04% to 0.187% (Figure 9, Table VI).
- The increase of *Enterobacteriaceae* family from 0.28% to 93.70% (Figure 8, Table V). Three major genera explain this increase:
    - *Citrobacter* with a relative abundance of 75.90% after the one-week challenge (Figure 9, Table VI).
    - *Escherichia-Shigella* with a relative abundance of 16.43% at "J+7" (Figure 9, Table VI).
    - *Klebsiella pneumoniae* which has a relative abundance of 1.10% after the intoxication with HGA (Figure 9, Table VI).

2.3.SHORT CHAIN FATTY ACIDS ANALYSIS

SCFAs are carboxylic acids with the presence of an aliphatic tail of two to six carbons. Just after the stabilization period, the three mainly SCFA (acetate, propionate and butyrate) are present in each colon with the following *ratio*: acetate > propionate> butyrate. Their concentrations are the highest in DC.

During the one-week challenge with HGA in AC, the concentration of acetate and butyrate decreased but the concentration in propionate remained rather stable. The concentrations are listed as follows: acetate goes from 2801 to 2173 mg/L, propionate goes from 2390 to 2318 mg/L and butyrate goes from 1388 to 1039 mg/L

It is interesting to note that, at the end of the challenge, there is an inversion between acetate and propionate. Indeed, at this time, the concentration of propionate is superior to that of acetate. This phenomenon stops two days after stopping the administration of HGA.

In the TC, an inversion between the concentration of acetate and propionate is noted in the beginning of the HGA administration (“J+2”). Indeed, the concentration between the day called “J” and the day called “J+2” varies as follows:

- Acetate goes from 1777 to 1386 mg/L
- Propionate goes from 1307 to 1700 mg/L

This shift is maintained thereafter even after stopping administration of the toxin.

In the DC, at the end of the challenge, the general production of the three SCFA decreased. The inversion between acetate and propionate is also present from the last day of challenge and is maintained after stopping the administration of HGA. The concentration of the different SCFA varies from day J to day J+7 as follows:

- Acetate goes from 3012 to 1887 mg/L,
- Propionate goes from 2551 to 2065 mg/L
- Butyrate goes from 1487 to 1092 mg/L

After the intoxication with HGA, the general production of acetate in the three colons decreases, while propionate seems to be more stable. The butyrate seems to be less impacted by the addition of HGA (Figure 10, Table VII).

TABLE IV: RELATIVES VALUES IN PERCENTAGE OF THE DIFFERENT BACTERIAL PHYLA IN EACH COLON COMPARTMENT

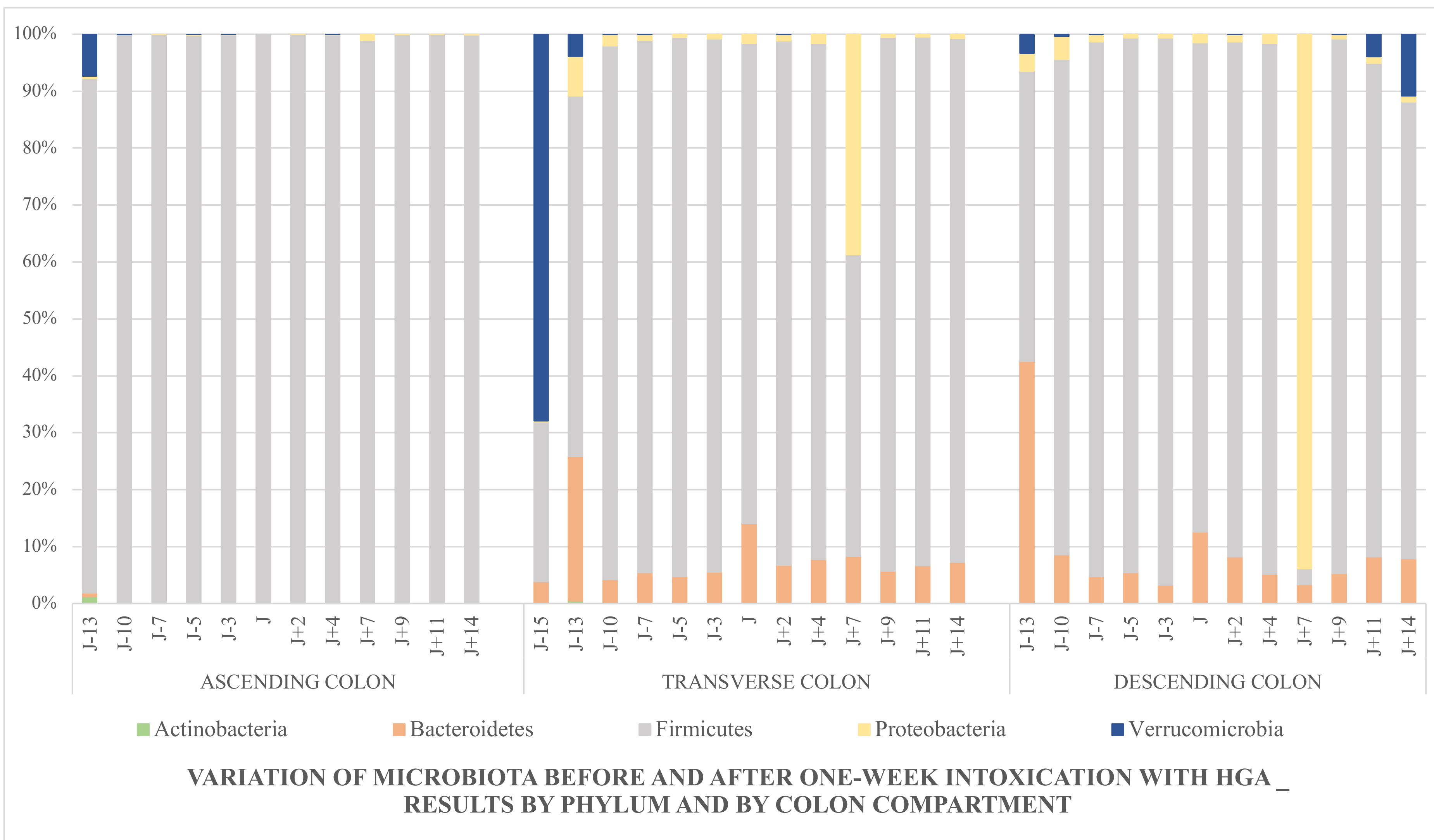
valeur < 0,5	ASCENDING COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroidetes	**0,600%**	0,023%	0,025%	0,000%	0,010%	0,000%	0,041%	0,053%	0,033%	0,011%	0,034%	0,036%
Firmicutes	**90,373%**	**99,954%**	**99,950%**	**99,830%**	**99,918%**	**100,000%**	**99,904%**	**99,869%**	**98,875%**	**99,966%**	**99,932%**	**99,915%**
Proteobacteria	0,496%	0,000%	0,025%	0,144%	0,051%	0,000%	0,041%	0,066%	**1,049%**	0,011%	0,034%	0,049%

	TRANSVERSE COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroidetes	**25,367%**	**4,216%**	**5,445%**	**4,788%**	**5,549%**	**14,094%**	**6,772%**	**7,794%**	**8,311%**	**5,691%**	**6,645%**	**7,223%**
Firmicutes	**63,292%**	**93,744%**	**93,448%**	**94,673%**	**93,686%**	**84,325%**	**92,057%**	**90,603%**	**52,978%**	**93,761%**	**92,883%**	**92,050%**
Proteobacteria	**7,021%**	**1,971%**	**1,049%**	**0,540%**	**0,753%**	**1,581%**	**1,147%**	**1,591%**	**38,680%**	**0,548%**	0,460%	**0,690%**

	DESCENDING COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroidetes	**42,315%**	**8,351%**	**4,743%**	**5,415%**	**3,296%**	**12,619%**	**8,264%**	**5,218%**	**3,327%**	**5,230%**	**8,198%**	**7,911%**
Firmicutes	**50,948%**	**87,080%**	**93,917%**	**93,949%**	**96,081%**	**85,919%**	**90,449%**	**93,219%**	**2,829%**	**93,963%**	**86,771%**	**80,194%**
Proteobacteria	**3,214%**	**3,978%**	**1,274%**	**0,625%**	**0,623%**	**1,452%**	**1,264%**	**1,562%**	**93,813%**	**0,739%**	**1,112%**	**1,103%**

The day called "J" is the day where the nutritional medium was intoxicated with HGA after taking the samples. It represents the end of the stabilization period. The days J+2 to J+7 represent the challenge with the introduction of HGA. The days J+9 to J+14 represent the week after the challenge. The data values which are > 0.5% are written in bold.

FIGURE 7



Relative values of microbiota phyla in percentage. The days called J-15 to J-2 represent the stabilization period. The day J is the day where the nutritional medium was intoxicated with HGA after taking the samples. The days J+2 to J+7 represent the challenge with HGA. The days J+9 to J+14 represent the week after the challenge.

TABLE V: RELATIVES VALUES IN PERCENTAGE OF THE DIFFERENT BACTERIAL FAMILY IN EACH COLON COMPARTMENT

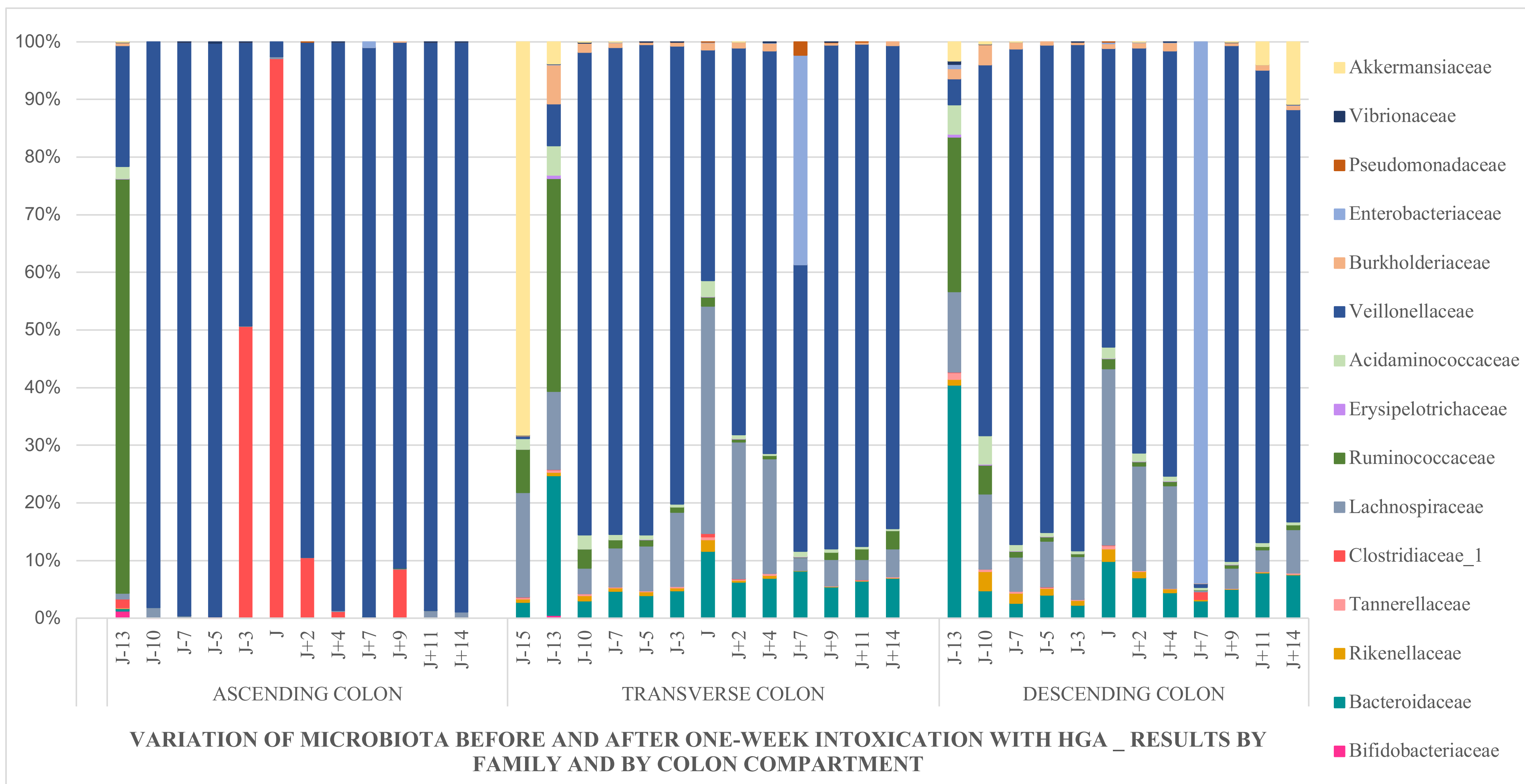
valeur < 0,5	ASCENDING COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroidaceae	0,339%	0,012%	0,000%	0,000%	0,010%	0,000%	0,027%	0,053%	0,011%	0,011%	0,023%	0,024%
Clostridiaceae_1	**1,409%**	0,000%	0,163%	0,262%	**50,664%**	**97,118%**	**10,499%**	**1,170%**	0,120%	**8,530%**	0,068%	0,170%
Lachnospiraceae	**0,861%**	**1,854%**	0,213%	0,013%	0,072%	0,292%	0,000%	0,171%	0,011%	0,124%	**1,257%**	**0,910%**
Ruminococcaceae	**66,645%**	0,000%	0,013%	0,000%	0,010%	0,010%	0,000%	0,000%	0,011%	0,011%	0,011%	0,000%
Veillonellaceae	**19,476%**	**98,054%**	**99,550%**	**99,542%**	**49,162%**	**2,539%**	**89,282%**	**98,515%**	**98,689%**	**91,245%**	**98,528%**	**98,836%**
Enterobacteriaceae	0,013%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	**0,939%**	0,000%	0,000%	0,000%

	TRANSVERSE COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroidaceae	**24,150%**	**2,956%**	**4,675%**	**3,961%**	**4,727%**	**11,587%**	**6,229%**	**6,955%**	**8,186%**	**5,417%**	**6,433%**	**6,927%**
Clostridiaceae_1	0,045%	0,034%	0,012%	0,011%	0,000%	**0,592%**	0,048%	0,025%	0,000%	0,024%	0,047%	0,000%
Lachnospiraceae	**13,507%**	**4,411%**	**6,786%**	**7,715%**	**12,836%**	**39,365%**	**23,708%**	**19,817%**	**2,210%**	**4,584%**	**3,570%**	**4,782%**
Ruminococcaceae	**36,782%**	**3,288%**	**1,352%**	**1,160%**	**0,869%**	**1,635%**	**0,531%**	**0,580%**	0,114%	**1,191%**	**1,803%**	**3,192%**
Veillonellaceae	**7,229%**	**83,456%**	**84,295%**	**84,960%**	**79,252%**	**39,946%**	**67,045%**	**69,737%**	**49,637%**	**87,272%**	**86,992%**	**83,742%**
Enterobacteriaceae	0,134%	0,000%	0,047%	0,000%	0,000%	0,065%	0,024%	0,049%	**36,242%**	0,012%	0,000%	0,000%

	DESCENDING COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroidaceae	**39,828%**	**4,523%**	**2,613%**	**4,030%**	**2,244%**	**9,864%**	**7,011%**	**4,426%**	**2,985%**	**5,025%**	**7,827%**	**7,562%**
Clostridiaceae_1	0,013%	0,023%	0,011%	0,011%	0,011%	0,074%	0,000%	0,000%	**1,389%**	0,000%	0,045%	0,000%
Lachnospiraceae	**13,877%**	**12,909%**	**5,918%**	**7,971%**	**7,419%**	**30,462%**	**18,153%**	**17,806%**	0,238%	**3,513%**	**3,751%**	**7,484%**
Ruminococcaceae	**26,556%**	**4,999%**	**1,010%**	**0,737%**	0,451%	**1,769%**	**0,700%**	**0,713%**	0,104%	**0,546%**	**0,561%**	**0,878%**
Veillonellaceae	**4,451%**	**63,918%**	**85,793%**	**84,426%**	**87,685%**	**51,579%**	**70,061%**	**73,772%**	**0,632%**	**89,268%**	**81,707%**	**71,326%**
Enterobacteriaceae	**0,766%**	0,023%	0,011%	0,000%	0,000%	0,275%	0,045%	0,079%	**93,699%**	0,000%	0,000%	0,011%

The day called "J" is the day where the nutritional medium was intoxicated with HGA after taking the samples. It represents the end of the stabilization period. The days J+2 to J+7 represent the challenge with the introduction of HGA. The days J+9 to J+14 represent the week after the challenge. The data values which are > 0.5% are written in bold

FIGURE 8



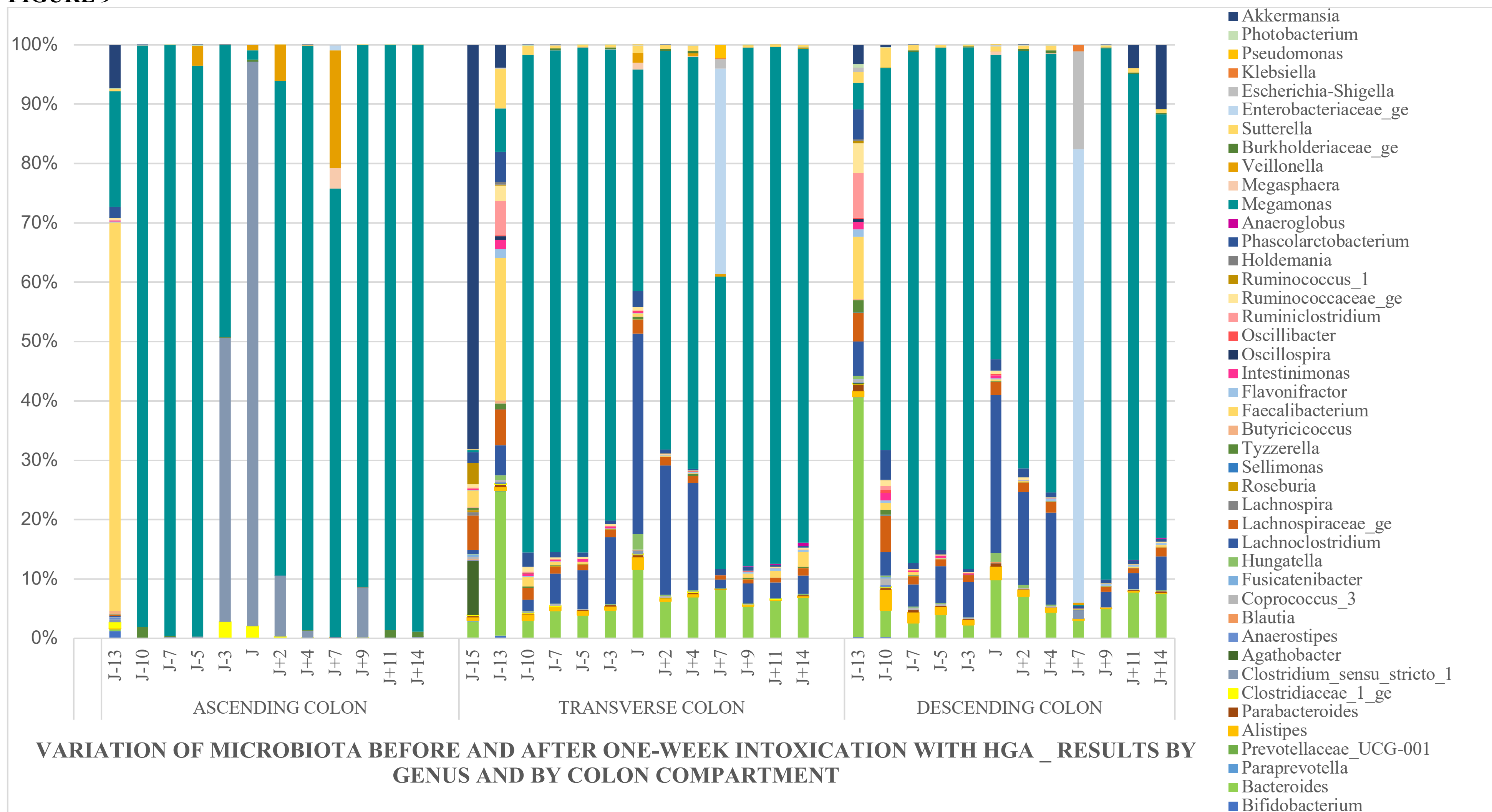
Relative values of microbiota family in percentage. The days called J-15 to J-2 represent the stabilization period. The day J is the day where the nutritional medium was intoxicated with HGA after taking the samples. The days J+2 to J+7 represent the challenge with HGA. The days J+9 to J+14 represent the week after the challenge.

TABLE VI: RELATIVES VALUES IN PERCENTAGE OF THE DIFFERENT BACTERIAL GENUS IN EACH COLON COMPARTMENT

Values<0,5%	ASCENDING COLON											
	J-13	J-10	J-7	J-5	J-3	J	J+2	J+4	J+7	J+9	J+11	J+14
Bacteroides	**0,339%**	0,012%	0,000%	0,000%	0,010%	0,000%	0,027%	0,053%	0,011%	0,011%	0,023%	0,024%
Clostridium_sensu_stricto_1	**0,522%**	0,000%	**0,163%**	**0,262%**	**47,814%**	**95,093%**	**10,280%**	**1,144%**	**0,120%**	**8,439%**	0,068%	**0,170%**
Lachnoclostridium	**0,157%**	0,012%	0,013%	0,000%	0,000%	0,010%	0,000%	0,026%	0,011%	0,034%	0,011%	0,012%
Megamonas	**19,423%**	**97,984%**	**99,537%**	**96,208%**	**49,162%**	**1,612%**	**83,224%**	**98,423%**	**75,486%**	**91,245%**	**98,517%**	**98,812%**
Megasphaera	0,000%	0,070%	0,000%	0,000%	0,000%	0,000%	0,027%	0,013%	**3,430%**	0,000%	0,011%	0,000%
Veillonella	0,000%	0,000%	0,013%	**3,334%**	0,000%	**0,927%**	**6,031%**	0,079%	**19,773%**	0,000%	0,000%	0,024%
Enterobacteriaceae_ge	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	**0,830%**	0,000%	0,000%	0,000%
Escherichia-Shigella	0,013%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	**0,109%**	0,000%	0,000%	0,000%
Klebsiella	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%
Pseudomonas	0,000%	0,000%	0,013%	0,000%	0,031%	0,000%	0,014%	0,000%	0,000%	0,000%	0,011%	0,000%
	TRANSVERSE COLON											
Bacteroides	**24,150%**	**2,956%**	**4,675%**	**3,961%**	**4,727%**	**11,587%**	**6,229%**	**6,955%**	**8,186%**	**5,417%**	**6,433%**	**6,927%**
Clostridium_sensu_stricto_1	0,000%	0,023%	0,000%	0,011%	0,000%	**0,581%**	0,024%	0,000%	0,000%	0,012%	0,035%	0,000%
Lachnoclostridium	**5,047%**	**1,868%**	**5,037%**	**6,533%**	**11,237%**	**33,739%**	**21,656%**	**18,005%**	**1,515%**	**3,429%**	**2,663%**	**3,081%**
Megamonas	**7,184%**	**83,364%**	**84,260%**	**84,925%**	**79,194%**	**37,138%**	**66,985%**	**69,182%**	**49,222%**	**87,201%**	**86,839%**	**83,064%**
Megasphaera	0,015%	0,023%	0,012%	0,023%	0,012%	**1,162%**	0,012%	0,012%	0,000%	0,000%	0,012%	0,025%
Veillonella	0,000%	0,000%	0,000%	0,000%	0,000%	**1,635%**	0,000%	**0,518%**	**0,415%**	0,000%	0,000%	**0,111%**
Enterobacteriaceae_ge	0,000%	0,000%	0,000%	0,000%	0,000%	0,043%	0,024%	0,000%	**34,571%**	0,012%	0,000%	0,000%
Escherichia-Shigella	**0,134%**	0,000%	0,023%	0,000%	0,000%	0,022%	0,000%	0,012%	**1,556%**	0,000%	0,000%	0,000%
Klebsiella	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	**0,114%**	0,000%	0,000%	0,000%
Pseudomonas	0,000%	0,000%	0,000%	0,023%	0,012%	0,022%	0,000%	0,000%	**2,283%**	0,036%	0,012%	0,000%
	DESCENDING COLON											
Bacteroides	**39,828%**	**4,523%**	**2,613%**	**4,030%**	**2,244%**	**9,864%**	**7,011%**	**4,426%**	**2,985%**	**5,025%**	**7,827%**	**7,562%**
Clostridium_sensu_stricto_1	0,000%	0,000%	0,011%	0,011%	0,011%	0,074%	0,000%	0,000%	**1,389%**	0,000%	0,045%	0,000%
Lachnoclostridium	**5,688%**	**3,909%**	**3,711%**	**6,241%**	**5,937%**	**26,467%**	**15,613%**	**15,474%**	**0,135%**	**2,569%**	**2,695%**	**5,739%**
Megamonas	**4,437%**	**63,837%**	**85,727%**	**84,414%**	**87,664%**	**51,038%**	**70,038%**	**73,749%**	**0,187%**	**89,256%**	**81,583%**	**71,089%**
Megasphaera	0,000%	0,000%	0,011%	0,000%	0,000%	**0,530%**	0,000%	0,023%	0,041%	0,011%	0,000%	0,000%
Veillonella	0,000%	0,000%	0,000%	0,000%	0,000%	0,011%	0,000%	0,000%	**0,404%**	0,000%	0,000%	0,000%
Enterobacteriaceae_ge	0,000%	0,012%	0,011%	0,000%	0,000%	**0,212%**	0,034%	0,034%	**76,174%**	0,000%	0,000%	0,011%
Escherichia-Shigella	**0,753%**	0,000%	0,000%	0,000%	0,000%	0,064%	0,000%	0,011%	**16,427%**	0,000%	0,000%	0,000%
Klebsiella	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,000%	0,011%	**1,099%**	0,000%	0,000%	0,000%
Pseudomonas	0,000%	0,000%	0,000%	0,000%	0,000%	0,011%	0,000%	0,000%	0,000%	0,000%	0,000%	0,056%

Relative values of microbiota family in percentage. The days called J-15 to J-2 represent the stabilization period. The day J is the day where the nutritional medium was intoxicated with HGA after taking the samples. The days J+2 to J+7 represent the challenge with HGA. The days J+9 to J+14 represent the week after the challenge.

FIGURE 9



Relative values of microbiota genus in percentage. The days called J-15 to J-2 represent the stabilization period. The day J is the day where the nutritional medium was intoxicated with HGA after taking the samples. The days J+2 to J+7 represent the challenge with HGA. The days J+9 to J+14 represent the week after the challenge.

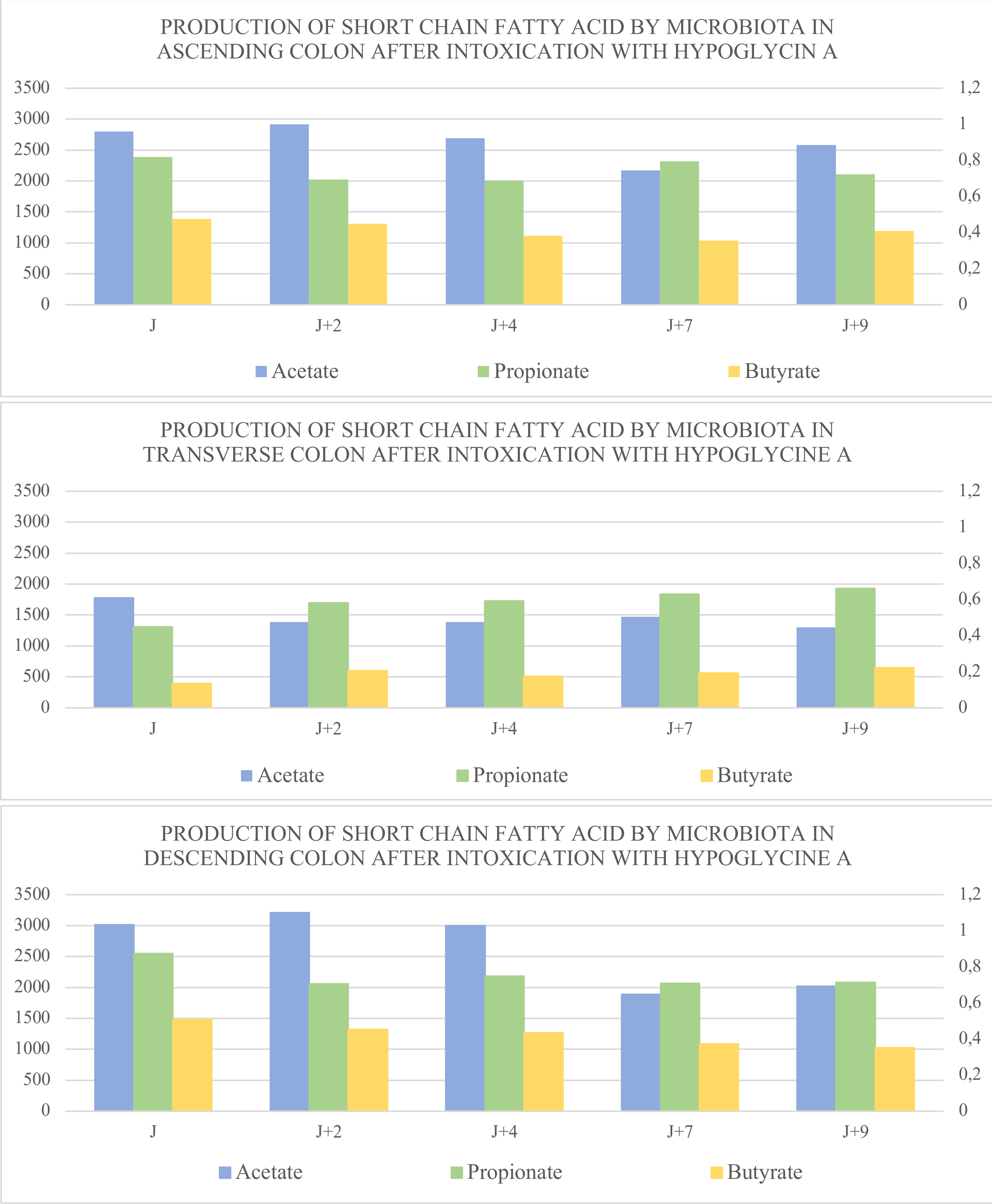
TABLE VII

Concentration of acetate, propionate and butyrate (mg/L) in ascending, transverse and descending colon

		C2	C3	C4
			mg/L	
J-13	AC	11339	1783	4576
J-13	TC	5192	749	1713
J-13	DC	3969	367	1547
J-10	AC	5073	1184	1959
J-10	TC	4133	1279	2024
J-10	DC	1941	1350	924
J-7	AC	3753	2601	2157
J-7	TC	2723	2120	1510
J-7	DC	1687	2363	535
J-5	AC	1303	2205	619
J-5	TC	2852	2549	1466
J-5	DC	2902	2303	1570
J-3	AC	2776	2532	1384
J-3	TC	1407	1984	547
J-3	DC	3150	2661	1502
J	AC	2801	2390	1388
J	TC	1777	1307	393
J	DC	3012	2551	1487
J+2	AC	2915	2028	1310
J+2	TC	1386	1700	604
J+2	DC	3208	2063	1322
J+4	AC	2694	2010	1116
J+4	TC	1385	1729	506
J+4	DC	3002	2184	1270
J+7	AC	2173	2318	1039
J+7	TC	1471	1838	566
J+7	DC	1887	2065	1092
J+9	AC	2582	2106	1192
J+9	TC	1300	1927	652
J+9	DC	2022	2086	1025
J+11	AC	2854	2481	1372
J+11	TC	3035	2705	1237
J+11	DC	1227	1831	446
J+14	AC	2468	2310	1132
J+14	TC	2645	2515	1189
J+14	DC	1136	2027	435

The days called “J-13” to “J” represent the stabilization period. The days “J+2” to “J+7” correspond to the challenge with intoxication with HGA. The days “J+9” to “J+14” represent the days after intoxication with HGA.

FIGURE 10



Concentration (mg/L) of acetate, propionate and butyrate produced by microbiota following intoxication with hypoglycin A in the three colon-compartments from the simulator of the human intestinal microbial ecosystem. The day J is the day where the nutritional medium was changed and intoxicated with HGA after taking the samples. The days J+2 to J+7 belong to the one-week challenge. The days J+9 represent the stop of intoxication.

DISCUSSION

Microbiota is mainly composed by bacteria and only by 0.5 % eukaryotes, 0.8% archea, 5.8% viruses and 0.2 to 0.3% fungi (Arumugam et al., 2011; Dieterich et al., 2018). The majority of microorganisms reside in the distal part of the digestive tract (> 10^{11} cells per gram content) (Singh et al., 2017).
The microbiota of child is a *Bifidobacterium*-dominated microbiota because of its milk diet. At around 3-years, the diet is mainly composed of solid food and the microbiota is then similar to the one of an adult: a *Bacteroidetes* (aerobic gram negative) and *Firmicutes*(aerobic gram positive)-dominated microbiota (Cresci and Bawden, 2015; Dieterich et al., 2018; Singh et al., 2017). These two bacterial phyla dominate the human ecosystem as in at least 60 mammalian species (Ley et al., 2008).
In fact, 94,2% of the sequence of all African and European samples belong to the four most populated bacterial phyla: *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria* (Cresci and Bawden, 2015). As explained *Firmicutes* and *Bacteroidetes* are in the majority (>99%) on the contrary of *Actinobacteria*, *Proteobacteria* and some other phylum, which are in the minority inside human digestive tract (Dieterich et al., 2018; Falony et al., 2016; Rajilić-Stojanović and De Vos, 2014; Shetty et al., 2017). They are less important phyla in gastrointestinal tract comparable to *Verrucomicrobia* (Rajilić-Stojanović and De Vos, 2014).
There are six bacterial phyla present at the beginning of the experiment: *Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Protebacteria* and *Verrucomicrobia*. Three of them are mainly represented and in particular at the end of the stabilization period: *Firmicutes* (average 90.08 % in the three colon), *Bacteroidetes* (average 8.9 % in the three colon) and *Protebacteria* (average 1.01 % in the three colon). This data corresponds to the information in the scientific literature about human adult microbiota.

The SHIME® requires a stabilization period depending on the normal residence time in the gastrointestinal tract. This residence time is 48 hours in a healthy adult male. Normally, a multiplication factor of 5 to 10 is recommended to allow the stabilization of microbiota: the time required therefore ranges from 10 to 20 days. To stabilize the production of SCFA, a duration of at least 15 days is needed (Van de Wiele et al., 2015). The stabilization time used in this experiment is 15 days which is sufficient for the production of SCFA but perhaps insufficient for the stabilization of the microbiota. In TC and DC, *Firmicutes* and *Bacteroidetes* phyla present a difference in relative abundance between the day "J-3" and the last day of the stabilization period. The *Firmicutes* phyla varies respectively from 93.68% to 84.33% in TC and from 96.08% to 85.92% in DC. The *Bacteroidetes* phyla varies from 5.55% to 14.10% in TC and from 3.29% to 12.62% in DC. Although the phyla present are those described in the scientific literature, the variations in relative abundances may be a sign of a lack of stability. To avoid this possible lack of stability, a longer period of stabilization should be required.

SCFAs are mainly product in the colon by gut microbiota thanks to owning the enzyme involved in their production (Guilloteau et al., 2010; Ríos-Covián et al., 2016; Tan et al., 2014). SCFA are produced by bacterial fermentation of dietary fiber in *a ratio* of 60% acetate (C2), 25% propionate (C3) and 15% butyrate (C4) (Tazoe et al., 2008). These three SCFA represent 90-95% of SCFA present in the colon. As the proportion of C2, C3 and C4 at the end of the stabilization period respect the proportion 60-25-15 and as their concentrations are stable between day "J-3" and day "J", the system seems to be stable before the beginning of the challenge. Therefore, the observed effects are probably due to the action of the toxin.

Following the intoxication with HGA, important changes in microbial population are noted. These changes highlight a dysbiosis after the introduction of HGA especially in TC and DC. The dysbiosis, a disease-promoting imbalance, occurs (1) when there is a loss or a decrease in microbial diversity which can promote the expansion of specific bacterial taxa or (2) when there are large shifts in the ratio between majority phyla and minority phyla or (3) when the expansion of new bacterial phylum or genus leads to an imbalance This imbalance is disease-promoting (Weiss and Hennet, 2017).
This dysbiosis can be highlighted by (1) the variation in the diversity indices (2) the increase of some particular bacterial populations and (3) the general decrease in the concentration of the different SCFA which demonstrate suffering of the microbiota.

About diversity indices, especially in TC and DC, the three indices show a loss of bacterial diversity. After the intoxication with HGA, this *Enterobacteriaceae* family sharply increase in TC and DC : the apparition of this family is a sign of dysbiosis and metabolic disease (Serino, 2018). This general dysbiosis was partially resolved one week after cessation of the HGA challenge.
In AC, there is a lack of bacterial diversity in the beginning but during the experiment, the indices describe an increase in diversity. This lack of diversity can explain the higher possibility in diversification inside the microbiota and the sudden appearance of *Clostridium butyricium* at the end of the stabilization period ("J-3") in place of *Megamonas*. This observation can mean the existence of a competition between these two genera. Another hypothesis is that *Clostridium butyricium* needs more anaerobic conditions and so need time for implantation.

SCFA are normally produced by microbiota and their concentration is correlated with the different population present in the microbiota. Butyrate is the energy substrate for colonocyte and produce 70% of the energy obtained by intestinal cells : it is the energy substrate for colonocyte and therefore has a positive effect on intestinal cells (Detman et al., 2019; Leblanc et al., 2017; Tan et al., 2014). It presents anticarcinogenic and anti-inflammatory properties (Rajilić-Stojanović and De Vos, 2014). Acetate and propionate are the energy substrate for peripheral tissues after reaching the liver through the portal vein (Leblanc et al., 2017; Tan et al., 2014). Propionate has an anti-inflammatory potential (Rajilić-

Stojanović and De Vos, 2014). The SCFA play a role in colonic blood flow, fluid/electrolyte uptake, colonic motility, ion transport …(Tazoe et al., 2008).
In AC, after HGA intoxication, there are two major observations: (1) the concentration of acetate and butyrate decrease but the concentration of propionate remains stable and (2) the proportion in acetate and propionate is inverted at the end of the challenge for several days. In this part of the gastrointestinal tract, after the intoxication with HGA, *Clostridium butyricum* decreases in the opposition to *Megamonas* and *Veillonella* genera that increase. The members of the genus *Clostridium* are able to use acetate and lactate to produce butyrate. The *Megamonas* and *Veillonella* genera are assacharolytic and produce propionate from end-products of sugar metabolisms of other bacteria. These observations corroborate the relative stability of propionate and the decrease in butyrate and acetate (Rajilić-Stojanović and De Vos, 2014).
In TC and DC, there is (1) an important decrease in concentration of each SCFA and (2) a shift between acetate and propionate with an increase of propionate and a decrease of acetate. This shift is maintained even after stopping administration of the toxin. The *Lachnospiraceae* family, that is butyrate-producer, sharply decreases to disappear after the introduction of HGA. The increase of the *Megamonas* genus can explain the increase in propionate and the maintaining of this shift after stopping the distribution of HGA (Rajilić-Stojanović and De Vos, 2014).

As described, clinical signs in human intoxicated with HGA include digestive clinical signs and severe hypoglycemia (Bressler et al., 1969). The population of the phyla *Proteobacteria* increase ion TC and DC after the intoxication with HGA. This increasing phylum is mainly represented by different families during this experiment: *Enterobacteriaceae* (with genera *Citrobacter, Escherichia-Shigella* and *Klebsiella*) and *Pseudomonadaceae* (with genus *Pseudomonas*). Some of these genera are able to cause different digestive symptoms in human and could be the explanation of clinical signs in JVS.
The genus *Citrobacter* is particularly increased and includes *C.rodentium* : this species is used to model several human intestinal disorders and causes pronounced dysbiosis with a reduction of diversity (Collins et al., 2014).
Escherichia Coli is involved in diarrheal and extraintestinal diseases (urinary tract, bloodstream and central nervous system) (Croxen et al., 2013) and is one marker of inflammatory processes with a decline in positive commensal bacteria which provide a protective effect on intestine (Tyakht et al., 2018).
Shigella leads to shigellosis with watery diarrhea, abdominal cramps and fever. This dysentery is caused by its ability to penetrate and colonize the colonic epithelium with a loss of barrier function and the presence of digestive inflammation (Mattock and Blocker, 2017).
Pseudomonas aeruginosa doesn't cause digestive clinical signs but its presence in human intestine before the admission in intensive care unit is associated with a 15 times increased risk of subsequent infection (von Klitzing et al., 2017).

Concerning the stability of the toxin in the feed, there are two major observations: (1) the concentration of HGA decreases in the feed during the week of challenge but the presence of pancreatic juice for 90 minutes doesn't have any effect on the concentration of HGA and (2) there is the presence of a peak compatible with HGA in the feed before the adding of HGA.
This peak compatible with HGA in the feed before adding the toxin could be explain by the presence of proteose peptone in the feed. In the HTPLC method describe in this study, the measure of HGA is possible *via* the detection of amine function (Sander et al., 2016). Therefore, if there are substances with amine group in the feed, these substances can be confused with HGA with this HTPLC method. In proteose peptone, there are amino acid and so there are amine groups able to confuse their own peak on HTPLC with the peak for HGA. Moreover, with chromatography technique, leucine and HGA are confused and there is no distinction between these two compounds (Carlier et al., 2015). The method used is not enough specific for the quantification of HGA.
The decrease of HGA in the feed is accompanied with a general decrease of the other substance present in the feed. Indeed, all the peaks decrease during the week. It seems that there is a general deterioration of the different components inside the feed and at least of amine group.
As explained, some bacteria are able to degrade protein and amino acid. Do the bacteria degrade amino acid compound in the nutritional medium? Do the bacteria degrade HGA during the one-week challenge? To explore theses hypotheses, a more specific method of quantification of HGA is needed and the presence of bacteria able to degrade HGA has to be demonstrate.
This decrease of the peak of HGA suggests a decrease of its concentration during the one-week challenge in SHIME®. To the author's knowledge, there is not information about the time needed to observe change in microbiota after administration of HGA. On inbred mice, after a shift in the macronutrients, microbiome can change in 24 hours. In human, dietary interventions can change microbiota on weeks to months (David et al., 2014). In the light of these observations, other experiment of intoxication with a daily administration of HGA should be performed to observe the change in microbiota.

In a preliminary study that compares the microbiota of horses with AM and healthy co-grazers, the microbiota of horses with AM show a significant decrease in *Lachnospiraceae* family. This information corroborates the results in this experiment (Cerri et al., 2017). Cerri et al., asked themselves about the presence of this bacterial family: is the family present only inside horses which develop the disease?
In this paper, the intoxication with HGA on a normal healthy microbiota leads to the same decrease of *Lachnospiraceae* family. This observation is in favor of a role of witness of the microbiota.

CONCLUSION

Changes in microbiota are observed despite the probable lack of stability of HGA in the nutritional medium of the SHIME®. A more specific method of quantification of HGA is needed. No statistical analyze was performed because of the lack of repetition of this experiment. In order to increase the statistical power and to verify the modification observed during this challenge with HGA further experiments need to be done.

BIBLIOGRAPHY

Adeva-Andany, M.M., López-Maside, L., Donapetry-García, C., Fernández-Fernández, C., Sixto-Leal, C., 2017. Enzymes involved in branched-chain amino acid metabolism in humans. Amino Acids 49, 1005–1028. https://doi.org/10.1007/s00726-017-2412-7

Allen, H.K., Bayles, D.O., Looft, T., Trachsel, J., Bass, B.E., Alt, D.P., Bearson, S.M.D., Nicholson, T., Casey, T.A., 2016. Pipeline for amplifying and analyzing amplicons of the V1-V3 region of the 16S rRNA gene. BMC Res. Notes 9, 1–6. https://doi.org/10.1186/s13104-016-2172-6

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.-M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Bjørn Nielsen, H., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E.G., Wang, J., Guarner, F., Pedersen, O., De Vos, W.M., Brunak, S., Doré, J., Consortium, M., Weissenbach, J., Dusko Ehrlich, S., 2011. Enterotypes of the human gut microbiome. Nature 473, 174–180. https://doi.org/10.1038/nature09944

Billington, D., Sherratt, H., 1981. Hypoglycin and metabolically related inhibitors. Methods Enzymol. 72, 610–6.

Blake, O.A., Bennink, M.R., Jackson, J.C., 2006. Ackee (Blighia sapida) hypoglycin A toxicity: Dose response assessment in laboratory rats. Food Chem. Toxicol. 44, 207–213. https://doi.org/10.1016/j.fct.2005.07.002

Blake, O.A., Jackson, J.C., Jackson, M.A., Gordon, C.L.A., 2004. Assessment of dietary exposure to the natural toxin hypoglycin in ackee (Blighia sapida) by Jamaican consumers. Food Res. Int. 37, 833–838. https://doi.org/10.1016/j.foodres.2004.05.003

Bochnia, M., Sander, J., Ziegler, J., Terhardt, M., Sander, S., Janzen, N., Cavalleri, J.M. V., Zuraw, A., Wensch-Dorendorf, M., Zeyner, A., 2019. Detection of MCPG metabolites in horses with atypical myopathy. PLoS One 14, e0211698. https://doi.org/10.1371/journal.pone.0211698

Bressler, R., Corredor, C., Brendel, K., 1969. Hypoglycin and hypoglycin-like compounds, in: Pharmacological Review. pp. 105–130.

Brooks, S.E., Audretsch, J.J., 1971. Hypoglycin toxicity in rats. II. Modification by riboflavin of mitochondrial changes in liver. Am. J. Pathol. 62, 309–20.

Brosnan, J.T., Brosnan, M.E., 2006. Branched-Chain Amino Acids: Metabolism, Physiological Function, and Application.

Brown, M., Bates, R.P., McGowan, C., Cornell, J.A., 1991. Influence of fruit maturity on the hypoglycin A level in ackee (Blighia Sapida). J. Food Saf. 12, 167–177. https://doi.org/10.1111/j.1745-4565.1991.tb00075.x

Bunert, C., Langer, S., Votion, D., Boemer, F., Anja, M., Ternes, K., Liesegang, A., 2018. Atypical myopathy in Père David ' s deer (Elaphurus davidianus) associated with ingestion of hypoglycin A 1–11. https://doi.org/10.1093/jas/sky200

Carlier, J., Guitton, J., Moreau, C., Boyer, B., Bévalot, F., Fanton, L., Habyarimana, J.A., Gault, G., Gaillard, Y., 2015. A validated method for quantifying hypoglycin A in whole blood by UHPLC-HRMS/MS. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 978–979, 70–77. https://doi.org/10.1016/j.jchromb.2014.11.029

Cerri, S., Taminiau, B., Amory, H., Daube, G., 2017. Comparaison of faecal microbiota of horses suffering from atypical myopahty and healthy co-grazers, in: Farah Day 2017. pp. 1–10.

Chen, K.K., Anderson, C., Mccowen, C., Harris, P.N., 1956. Pharmacologic Action og Hypoglycin A and B. J. Pharmacol. Exp. Ther. 121, 272–285.

Collins, J.W., Keeney, K.M., Crepin, V.F., Rathinam, V.A.K.K., Fitzgerald, K.A., Finlay, B.B., Frankel, G., 2014. Citrobacter rodentium: Infection, inflammation and the microbiota. Nat. Rev. Microbiol. 12, 612–623. https://doi.org/10.1038/nrmicro3315

Cresci, G.A., Bawden, E., 2015. Gut Microbiome: What We Do and Don't Know. Nutr. Clin. Pract. 30, 734–46. https://doi.org/10.1177/0884533615609899

Croxen, M.A., Law, R.J., Scholz, R., Keeney, K.M., Wlodarska, M., Finlay, B.B., 2013. Recent Advances in Understanding Enteric Pathogenic Escherichia coli. Clin. Microbiol. Rev. 26, 822–

880. https://doi.org/10.1128/CMR.00022-13
Dai, Z.L., Li, X.L., Peng-Bin, X., Zhang, J., Wu, G., Zhu, W.Y., 2012. Metabolism of select amino acids in bacteria from the pig small intestine. Amino Acids 42, 1597–1608. https://doi.org/10.1007/s00726-011-0846-x
Dai, Z.L., Wu, G., Zhu, W.Y., Zhao-Lai, D., Guoyao, W., Wei-Yun, Z., 2011. Amino acid metabolism in intestinal bacteria: links between gut ecology and host health. Front. Biosci. (Landmark Ed. 16, 1768–1786. https://doi.org/10.1016/S0002-8223(05)01189-2
Dai, Z.L., Zhang, J., Wu, G., Zhu, W.Y., 2010. Utilization of amino acids by bacteria from the pig small intestine. Amino Acids 39, 1201–1215. https://doi.org/10.1007/s00726-010-0556-9
Das, M., John, T.J., 2017. Lychee-associated acute hypoglycaemic encephalopathy outbreaks in Muzaffarpur, India, The Lancet Global Health. https://doi.org/10.1016/S2214-109X(17)30269-3
David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A. V, Devlin, A.S., Varma, Y., Fischbach, M.A., Biddinger, S.B., Dutton, R.J., Turnbaugh, P.J., 2014. Diet and reproducibly alters the humen gut micobiome. NIH Public Access 505, 559–563. https://doi.org/10.1038/nature12820.Diet
Davila, A.M., Blachier, F., Gotteland, M., Andriamihaja, M., Benetti, P.H., Sanz, Y., Tomé, D., 2013. Intestinal luminal nitrogen metabolism: Role of the gut microbiota and consequences for the host. Pharmacol. Res. 68, 95–107. https://doi.org/10.1016/j.phrs.2012.11.005
Detman, A., Mielecki, D., Chojnacka, A., Salamon, A., Błaszczyk, M.K., Sikora, A., 2019. Cell factories converting lactate and acetate to butyrate: Clostridium butyricum and microbial communities from dark fermentation bioreactors. Microb. Cell Fact. 18, 1–12. https://doi.org/10.1186/s12934-019-1085-1
Dickinson, J.R., Dawes, I.W., 2009. The catabolism of branched-chain amino acids occurs via 2-oxoacid dehydrogenase in Saccharomyces cerevisiae. J. Gen. Microbiol. 138, 2029–2033. https://doi.org/10.1099/00221287-138-10-2029
Dieterich, W., Schink, M., Zopf, Y., 2018. Microbiota in the Gastrointestinal Tract. Med. Sci. 6, 1–15. https://doi.org/10.3390/medsci6040116
Douny, C., Dufourny, S., Brose, F., Verachtert, P., Rondia, P., Lebrun, S., Marzorati, M., Everaert, N., Delcenserie, V., Scippo, M.L., 2019. Development of an analytical method to detect short-chain fatty acids by SPME-GC–MS in samples coming from an in vitro gastrointestinal model. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1124, 188–196. https://doi.org/10.1016/j.jchromb.2019.06.013
Falony, G., Joossens, M., Vieira-Silva, S., Wang, J., Darzi, Y., Faust, K., Kurilshikov, A., Bonder, M.J., Valles-Colomer, M., Vandeputte, D., Tito, R.Y., Chaffron, S., Rymenans, L., Verspecht, C., De Sutter, L., Lima-Mendez, G., D'hoe, K., Jonckheere, K., Homola, D., Garcia, R., Tigchelaar, E.F., Eeckhaudt, L., Fu, J., Henckaerts, L., Zhernakova, A., Wijmenga, C., Raes, J., 2016. Population-level analysis of gut microbiome variation.
Feng, P.C., Patrick, S.J., 1958. Studies of the action of hypoglycin A, a hypoglycaemic substance. Br. J. Pharmacol. Chemother. 13, 125.
Fowden, L., Pratt, H.M., 1973. Cyclopropylamino acids of the genus Acer: Distribution and biosynthesis. Phytochemistry 12, 1677–1681. https://doi.org/10.1016/0031-9422(73)80387-5
Gonzalez-Medina, S., Hyde, C., Lovera, I., Piercy, R.J., 2018. Detection of equine atypical myopathy-associated hypoglycin a in plant material: Optimisation and validation of a novel lc-ms based method without derivatisation. PLoS One 13, 13–15. https://doi.org/10.1371/journal.pone.0199521
Gray, D.O., Fowden, L., 1962. α-(Methylenecyclopropyl)glycine from Litchi seeds. Biochem. J. 82, 385–389. https://doi.org/10.1042/bj0820385
Guilloteau, P., Martin, L., Eeckhaut, V., Ducatelle, R., Zabielski, R., Van Immerseel, F., 2010. From the gut to the peripheral tissues: the multiple effects of butyrate. Nutr. Res. Rev. 23, 366–384. https://doi.org/10.1017/s0954422410000247
Habyarimana, J.A., Baise, E., Douny, C., Weber, M., Boemer, F., De Tullio, P., Franck, T., Marcillaud Pitel, C., Frederich, M., Mouithys-Mickalad, A., Richard, E., Scippo, M.-L., Votion, D., Gustin, P., 2017. Development of an HPTLC method for determination of hypoglycin A in aqueous extracts of seedlings and samaras of Acer species. https://doi.org/10.1101/148262
Harper, A.E., Miller, R.H., Block, K.P., 1984. Branched-Chain Amino Acid Metabolism. Annu. Rev.

Nutr. 4, 409–454. https://doi.org/10.1146/annurev.nu.04.070184.002205
Hassal, C.H., Reyle, K., 1954. HypoglycinA and B , two biologically active polypeptides drom Blighia sapida. Biochem. J. 60, 334–339.
Isenberg, S.L., Carter, M.D., Graham, L.A., Mathews, T.P., Johnson, D., Thomas, J.D., Pirkle, J.L., Johnson, R., 2015. Quantification of Metabolites for Assessing Human Exposure to Soapberry Toxins Hypoglycin A and Methylenecyclopropylglycine. Chem. Res. Toxicol. 28, 1753–1759. https://doi.org/10.1021/acs.chemrestox.5b00205
Isenberg, S.L., Carter, M.D., Hayes, S.R., Graham, L.A., Mathews, T.P., Harden, L.A., Takeoka, G.R.R., Thomas, J.D.D., Pirkle, J.L., Johnson, R.C., Johnson, D., Mathews, T.P., Harden, L.A., Takeoka, G.R.R., Thomas, J.D.D., Pirkle, J.L., Johnson, R.C., Carter, M.D., 2017. Quantification of Toxins in Soapberry (Sapindaceae) Arils: Hypoglycin A and Methylenecyclopropylglycine. J. Agric. Food Chem. 64, 5607–5613. https://doi.org/10.1021/acs.jafc.6b02478.Quantification
Joskow, R., Belson, M., Vesper, H., Backer, L., Rubin, C., 2006. Ackee fruit poisoning: An outbreak investigation in Haiti 2000-2001, and review of the literature. Clin. Toxicol. 44, 267–273. https://doi.org/10.1080/15563650600584410
Khatra, B.S., Chawla, R.K., Sewell, C.W., Rudman, D., 1977. Distribution of branched-chain α-keto acid dehydrogenases in primate tissues. J. Clin. Invest. 59, 558–564. https://doi.org/10.1172/JCI108671
Leblanc, J.G.J., Chain, F., Martín, R., Bermúdez-Humarán, L.G., Courau, S., Langella, P., 2017. Beneficial effects on host energy metabolism of short-chain fatty acids and vitamins produced by commensal and probiotic bacteria. Microb. Cell Fact. 16, 79. https://doi.org/10.1186/s12934-017-0691-z
Ley, R.E., Hamady, M., Lozupone, C., Turnbaugh, P.J., Ramey, R.R., Bircher, J.S., Schlegel, M.L., Tucker, T.A., Schrenzel, M.D., Knight, R., Gordon, J.I., 2008. Evolution of mammals and their gut microbes. Science 320, 1647–51. https://doi.org/10.1126/science.1155725
Mailliard, M.E., Stevens, B.R., Mann, G.E., 1995. Amino Acid Transport by Small Intestinal, Hepatic, and Pancreatic Epithelia. Gastroenterology 108, 888–910. https://doi.org/10.1016/0016-5085(95)90466-2
Massey, L.K., Sokatch, J.R., Conrad, R.S., 1976. Branched-Chain Amino Acid Catabolism in Bacteria, Bacteriological reviews.
Mathew, J.L., John, T.J., Shrivastava, A., Kumar, A., Thomas, J.D., Laserson, K.F., Bhushan, G., Carter, M.D., 2017. Exploration of association between litchi consumption and seasonal acute encephalopathy syndrome. Indian Pediatr. 54, 319–325. https://doi.org/10.1007/s13312-017-1095-1
Matthews, D.M., 1971. Protein absorption. J. Clin. Pathol. S3-5, 29–40. https://doi.org/10.1136/jcp.s3-5.1.29
Mattock, E., Blocker, A.J., 2017. How Do the Virulence Factors of Shigella Work Together to Cause Disease? Front. Cell. Infect. Microbiol. 7, 1–24. https://doi.org/10.3389/fcimb.2017.00064
McCully, V., Burns, G., Sokatch, J.R., 2015. Resolution of branched-chain oxo acid dehydrogenase complex of Pseudomonas aeruginosa PAO. Biochem. J. 233, 737–742. https://doi.org/10.1042/bj2330737
Melde, K., Buettner, H., Boschert, W., Wolf, H.P.O., Ghisla, S., 1989. Mechanism of hypoglycaemic action of methylenecyclopropylglycine. Biochem. J. 259, 921–924. https://doi.org/10.1042/bj2590921
Melde, K., Jackson, S., Bartlett, K., Stanley, H., Sherratt, H., Ghisla, S., 1991. Metabolic consequences of methylenecyclopropylglycine poisoning in rats, Biochemical Journal. https://doi.org/10.1016/j.ceramint.2018.03.060
Molly, K., Vande Woestyne, M., Smet, I. De, Verstraete, W., Woestyne, M. Vande, Smet, I. De, Verstraete, W., Vande Woestyne, M., De Smet, I., Verstraete, W., 1994. Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. Microb. Ecol. Health Dis. 7, 191–200. https://doi.org/10.3109/08910609409141354
Neis, E., Dejong, C., Resnen, S., 2015. The Role of Microbial Amino Acid Metabolism in Host Metabolism. Nutrients 7, 2930–2946. https://doi.org/10.3390/nu7042930
Ngo, J., Taminiau, B., Fall, P.A., Daube, G., Fontaine, J., 2018. Ear canal microbiota – a comparison

between healthy dogs and atopic dogs without clinical signs of otitis externa. Vet. Dermatol. 29, 425-e140. https://doi.org/10.1111/vde.12674
Ohira, H., Tsutsui, W., Fujioka, Y., 2017. Are Short Chain Fatty Acids in Gut Microbiota Defensive Players for Inflammation and Atherosclerosis? J Atheroscler Thromb 24, 660–672. https://doi.org/10.5551/jat.RV17006
Possemiers, S., Verthé, K., Uyttendaele, S., Verstraete, W., Verth, K., Uyttendaele, S., Verstraete, W., 2004. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol. Ecol. 49, 495–507. https://doi.org/10.1016/j.femsec.2004.05.002
Rajilić-Stojanović, M., De Vos, W., 2014. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol. Rev. 38, 996–1047. https://doi.org/10.1111/1574-6976.12075
Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., De los Reyes-Gavilán, C.G., Salazar, N., Mijakovic, I., Sen, B., Ji, B., Salazar, N., Ríos-Covián, D., Ruas-Madiedo, P., Margolles, A., Gueimonde, M., De los Reyes-Gavilán, C.G., 2016. Intestinal short chain fatty acids and their link with diet and human health. Front. Microbiol. 7, 1–9. https://doi.org/10.3389/fmicb.2016.00185
Sander, J., Terhardt, M., Sander, S., Janzen, N., 2017. Quantification of methylenecyclopropyl compounds and acyl conjugates by UPLC-MS/MS in the study of the biochemical effects of the ingestion of canned ackee (Blighia sapida) and lychee (Litchi chinensis). J. Agric. Food Chem. 65, 2603–2608. https://doi.org/10.1021/acs.jafc.7b00224
Sander, J., Terhardt, M., Sander, S., Janzen, N., 2016. Quantification of hypoglycin A as butyl ester. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1029–1030, 169–173. https://doi.org/10.1016/j.jchromb.2016.07.005
Sanford, A.A., Isenberg, S.L., Carter, M.D., Mojica, M.A., Mathews, T.P., Harden, L.A., Takeoka, G.R., Thomas, J.D., Pirkle, J.L., Johnson, R., 2018. Quantitative HPLC–MS/MS analysis of toxins in soapberry seeds: Methylenecyclopropylglycine and hypoglycin A. Food Chem. 264, 449–454. https://doi.org/10.1016/j.foodchem.2018.04.093
Serino, M., 2018. Molecular Paths Linking Metabolic Diseases, Gut Microbiota Dysbiosis and Enterobacteria Infections. J. Mol. Biol. 430, 581–590. https://doi.org/10.1016/J.JMB.2018.01.010
Sherratt, H.S.A., 1986. Hypoglycin, the famous toxin of the unripe Jamaican ackee fruit. Trends Pharmacol. Sci. 7, 186–191. https://doi.org/10.1016/0165-6147(86)90310-X
Shetty, S.A., Lahti, L., Smidt, H., De Vos, W.M., 2017. Intestinal microbiome landscaping: insight in community assemblage and implications for microbial modulation strategies. FEMS Microbiol. Rev. 045, 182–199. https://doi.org/10.1093/femsre/fuw045
Shrivastava, A., Kumar, A., Thomas, J.D., Laserson, K.F., Bhushan, G., Carter, M.D., Chhabra, M., Mittal, V., Khare, S., Sejvar, J.J., Dwivedi, M., Isenberg, S.L., Johnson, R., Pirkle, J.L., Sharer, J.D., Hall, P.L., Yadav, R., Velayudhan, A., Papanna, M., Singh, P., Somashekar, D., Pradhan, A., Goel, K., Pandey, R., Kumar, M., Kumar, S., Chakrabarti, A., Sivaperumal, P., Kumar, A.R., Schier, J.G., Chang, A., Graham, L.A., Mathews, T.P., Johnson, D., Valentin, L., Caldwell, K.L., Jarrett, J.M., Harden, L.A., Takeoka, G.R., Tong, S., Queen, K., Paden, C., Whitney, A., Haberling, D.L., Singh, R., Singh, R.S., Earhart, K.C., Dhariwal, A.C., Chauhan, L.S., Venkatesh, S., Srikantiah, P., 2017. Association of acute toxic encephalopathy with litchi consumption in an outbreak in Muzaffarpur, India, 2014: a case-control study. Lancet Glob. Heal. 5, e458–e466. https://doi.org/10.1016/S2214-109X(17)30035-9
Singh, R.K., Chang, H.-W., Yan, D., Lee, K.M., Ucmak, D., Wong, K., Abrouk, M., Farahnik, B., Nakamura, M., Zhu, T.H., Bhutani, T., Liao, W., 2017. Influence of diet on the gut microbiome and implications for human health. J Transl Med 15, 1–17. https://doi.org/10.1186/s12967-017-1175-y
Singh, V.K., Hattangady, D.S., Giotis, E.S., Singh, A.K., Chamberlain, N.R., Stuart, M.K., Wilkinson, B.J., 2008. Insertional inactivation of branched-chain α-keto acid dehydrogenase in Staphylococcus aureus leads to decreased branched-chain membrane fatty acid content and increased susceptibility to certain stresses. Appl. Environ. Microbiol. 74, 5882–5890. https://doi.org/10.1128/AEM.00882-08

Stoll, B., Henry, J., Reeds, P.J., Yu, H., Burrin, D.G., 1997. Nutrient Metabolism Catabolism Dominates the First-Pass Intestinal Metabolism of Dietary Essential Amino Acids in Milk Protein-Fed Piglets 1,2.

Tan, J., Mckenzie, C., Potamitis, M., Thorburn, A.N., Mackay, C.R., Macia, L., 2014. The Role of Short-Chain Fatty Acids in Health and Disease, in: Advances in Immunology. pp. 91–119. https://doi.org/10.1016/B978-0-12-800100-4.00003-9

Tanaka, K., 1972. On the Mode of Action of Hypoglycin A, The Journal of Biological Chemistry.

Tanaka, K., Kean, E.A., Johnson, B., 1976. Jamaican Vomiting Sickness. N. Engl. J. Med. 295, 461–467. https://doi.org/10.1056/NEJM197608262950901

Tanaka, K., Miller, E.M., Isselbacher, K.J., 1971. Hypoglycin A: a specific inhibitor of isovaleryl CoA dehydrogenase. Proc. Natl. Acad. Sci. U. S. A. 68, 20–4. https://doi.org/10.1073/pnas.68.1.20

Tazoe, H., Otomo, Y., Kaji, I., Tanaka, R., Karaki, S.-I., Kuwahara, A., 2008. Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions. Artic. J. Physiol. Pharmacol. an Off. J. Polish Physiol. Soc. 59, 251–262.

Tyakht, A. V., Manolov, A.I., Kanygina, A. V., Ischenko, D.S., Kovarsky, B.A., Popenko, A.S., Pavlenko, A. V., Elizarova, A. V., Rakitina, D. V., Baikova, J.P., Ladygina, V.G., Kostryukova, E.S., Karpova, I.Y., Semashko, T.A., Larin, A.K., Grigoryeva, T. V., Sinyagina, M.N., Malanin, S.Y., Shcherbakov, P.L., Kharitonova, A.Y., Khalif, I.L., Shapina, M. V., Maev, I. V., Andreev, D.N., Belousova, E.A., Buzunova, Y.M., Alexeev, D.G., Govorun, V.M., 2018. Genetic diversity of Escherichia coli in gut microbiota of patients with Crohn's disease discovered using metagenomic and genomic analyses. BMC Genomics 19, 1–14. https://doi.org/10.1186/s12864-018-5306-5

Valberg, S.J., Sponseller, B.T., Hegeman, A.D., Earing, J., Bender, J.B., Martinson, K.L., Patterson, S.E., Sweetman, L., 2013. Seasonal pasture myopathy/atypical myopathy in North America associated with ingestion of hypoglycin A within seeds of the box elder tree. Equine Vet. J. 45, 419–426. https://doi.org/10.1111/j.2042-3306.2012.00684.x

Van de Wiele, T., Van den Abbeele, P., Ossieur, W., Possemiers, S., Marzorati, M., 2015. The Simulator of the Human Intestinal Microbial Ecosystem (SHIME ®), in: The Impact of Food Bio-Actives on Gut Health,. pp. 305–317. https://doi.org/10.1007/978-3-319-16104-4

Van Galen, G., Marcillaud Pitel, C., Saegerman, C., Patarin, F., Amory, H., Baily, J.D., Cassart, D., Gerber, V., Hahn, C., Harris, P., Keen, J.A., Kirschvink, N., Lefere, L., Mcgorum, B., Muller, J.M. V, Picavet, M.T.J.E., Piercy, R.J., Roscher, K., Serteyn, D., Unger, L., van der Kolk, J.H., van Loon, G., Verwilghen, D., Westermann, C.M., Votion, D., 2012. European outbreaks of atypical myopathy in grazing equids (2006-2009): Spatiotemporal distribution, history and clinical features. Equine Vet. J. 44, 614–620. https://doi.org/10.1111/j.2042-3306.2012.00556.x

van Galen, G., Saegerman, C., Marcillaud Pitel, C., Patarin, F., Amory, H., Baily, J.D., Cassart, D., Gerber, V., Hahn, C., Harris, P., Keen, J.A., Kirschvink, N., Lefere, L., Mcgorum, B., Muller, J.M. V, Picavet, M.T.J.E., Piercy, R.J., Roscher, K., Serteyn, D., Unger, L., van der Kolk, J.H., van Loon, G., Verwilghen, D., Westermann, C.M., Votion, D., 2012. European outbreaks of atypical myopathy in grazing horses (2006-2009): Determination of indicators for risk and prognostic factors. Equine Vet. J. 44, 621–625. https://doi.org/10.1111/j.2042-3306.2012.00555.x

Venema, K., Van den Abbeele, P., 2013. Experimental models of the gut microbiome. Best Pract. Res. Clin. Gastroenterol. 27, 115–126. https://doi.org/10.1016/j.bpg.2013.03.002

Von Holt, C., Chang, J., Von Holt, M., Böhm, H., 1964. Metabolism and metabolic effects of hypoglycin. Biochim. Biophys. Acta 0, 611–613.

von Klitzing, E., Ekmekciu, I., Bereswill, S., Heimesaat, M.M., 2017. Intestinal and systemic immune responses upon multi-drug resistant Pseudomonas aeruginosa colonization of mice harboring a human gut microbiota. Front. Microbiol. 8, 1–12. https://doi.org/10.3389/fmicb.2017.02590

Votion, D., 2018. Analysing hypoglycin A, methylenecyclopropylacetic acid conjugates and acylcarnitines in blood to confirm the diagnosis and improve our understanding of atypical myopathy. Equine Vet. Educ. 30, 29–30. https://doi.org/10.1111/eve.12617

Votion, D., Linden, A., Saegerman, C., Engels, P., Erpicum, M., Thiry, E., Delguste, C., Rouxhet, S., Demoulin, V., Navet, R., Sluse, F., Serteyn, D., Galen, G.V., Amory, H., 2007. History and clinical features of atypical myopathy in horses in Belgium (2000-2005). J. Vet. Intern. Med. 21,

1380–91. https://doi.org/10.1892/07-053.1
Votion, D., van Galen, G., Sweetman, L., Boemer, F., de Tullio, P., Dopagne, C., Lefere, L., Mouithys-Mickalad, A., Patarin, F., Rouxhet, S., van Loon, G., Serteyn, D., Sponseller, B.T., Valberg, S.J., 2014. Identification of methylenecyclopropyl acetic acid in serum of European horses with atypical myopathy. Equine Vet. J. 46, 146–149. https://doi.org/10.1111/evj.12117
Weiss, G.A., Hennet, T., 2017. Mechanisms and consequences of intestinal dysbiosis. Cell. Mol. Life Sci. 74, 2959–2977. https://doi.org/10.1007/s00018-017-2509-x
Wenz, A., Thorpe, C., Ghisla, S., 1981. Inactivation of general acyl-CoA dehydrogenase from pig kidney by a metabolite of hypoglycin A. J. Biol. Chem. 256, 9809–9812.
Westermann, C.M., Dorland, L., Votion, D., de Sain-van der Velden, M.G.M., Wijnberg, I.D., Wanders, R.J.A., Spliet, W.G.M., Testerink, N., Berger, R., Ruiter, J.P.N., van der Kolk, J.H., 2008. Acquired multiple Acyl-CoA dehydrogenase deficiency in 10 horses with atypical myopathy. Neuromuscul. Disord. 18, 355–364. https://doi.org/10.1016/j.nmd.2008.02.007
Yeaman, S.J., 1989. The 2-oxo acid dehydrogenase complexes: recent advances. Biochem. J. 257, 625–632. https://doi.org/10.1042/bj2570625
Zhang, J., Ding, X., Guan, R., Zhu, C., Xu, C., Zhu, B., Zhang, H., Xiong, Z., Xue, Y., Tu, J., Lu, Z., 2018. Evaluation of different 16S rRNA gene V regions for exploring bacterial diversity in a eutrophic freshwater lake. Sci. Total Environ. 618, 1254–1267. https://doi.org/10.1016/j.scitotenv.2017.09.228
Zhang, L.J., Fontaine, R.E., 2017. Lychee-associated encephalopathy in China and its reduction since 2000. Lancet Glob. Heal. 5, e865. https://doi.org/10.1016/S2214-109X(17)30291-7