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Ligands of the aryl hydrocarbon receptor produced by gut microbiota

Bachelor thesis

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Abstract

This study aimed to identify the compound(s) present in the microbial supernatant originating from SHIME® fermentation, that trigger the aryl hydrocarbon receptor (AhR). The AhR activation was measured using the CALUX® bioassay. HPLC was used to fractionate samples, identify compounds, and compare M-SHIME® supernatants originating from different colon parts: ascending, transverse, and descending colon. The AhR activation was found to increase from the ascending and the transverse colon to the descending colon. The tryptophan concentration was found to decrease from the ascending colon to the transverse and the descending colon, but tryptophan was found to be a minor AhR agonist. Like tryptophan, the AhR ligands present in the microbial supernatant were found to be polar. Another compound, which is indole, was also identified in the microbial supernatant, but was not found to activate AhR. No major AhR ligands are identified, but the results indicate that tryptophan derivatives are the main contributors to the activation of AhR in the microbial supernatant. Thus, the microbial supernatant originating from SHIME® fermentation seems to activate AhR due to tryptophan derivatives that are AhR agonists.

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1. Introduction

According to the World Health Organization, a healthy, balanced diet means that one should eat enough, but not too much calories [1]. They indicate that fruits, vegetables, legumes, nuts and whole grains should be consumed, of which at least 400g of fruits and vegetables a day. Besides, care must be taken with free sugars, (saturated and industrial trans) fats, and salt: less than 10% of the total energy intake should come from free sugars, 30% of the total energy intake should come from fats, and less than 5g of salt should be consumed each day.

A healthy diet helps to protect against malnutrition, as well as non-communicable diseases including diabetes, heart disease, stroke and cancer [1]. Poor nutrition also increases the risk of infections [2]. All this means that nutrition and health are closely related, so a healthy diet is of great importance. Still, many people do not have a healthy diet. In the Western society there are often too much calories consumed, and the diet is in many cases not well balanced [3, 4]. Overweight is a result of a positive energy balance, meaning that more energy is stored than utilised [5, 6]. Overweight is known to be a major contributor of causes of death [7]. However, undernutrition is also a problem, especially in Africa [8, 9].

So the diet is extremely important for the health, and a poor diet increases the risk on various diseases [1, 2]. Not only the diet is important, the gut microbiota play a large role in health as well, and also have connections with e.g. obesity and type 2 diabetes [10].

1.1. Gut microbiota

There are many bacteria present in the human body: according to Sender et al. (2016), the average person contains a comparable amount of bacteria and human cells [11]. This research states that the total number of bacteria in an average 70kg male is equal to $3.8*10^{13}$, while the total count of body cells of this male equals to $3.0*10^{13}$. Most of these bacteria are present in the intestines, especially in the large intestine [12]. Part of the human gastrointestinal tract can be seen in figure 1 [13]. Other locations in the body which houses bacteria are the mouth (dental plaque and saliva), the skin, and the stomach. The intestinal microbiota is currently thought to be composed of 500-1000 different species [12]. The biomass of these bacteria is about 0.2kg [11]. The composition of the microbiota differs per individual, and the diet is known to modulate the composition of the gut microbiota [14, 15]. The gut environment differs considerably between the different anatomical regions in terms of physiology, digesta, flow rates, substrate availability, host secretions, pH, and oxygen tension [16]. This is why the human intestinal microbiota should be viewed as a collection of semi-discrete communities, instead of one big community.

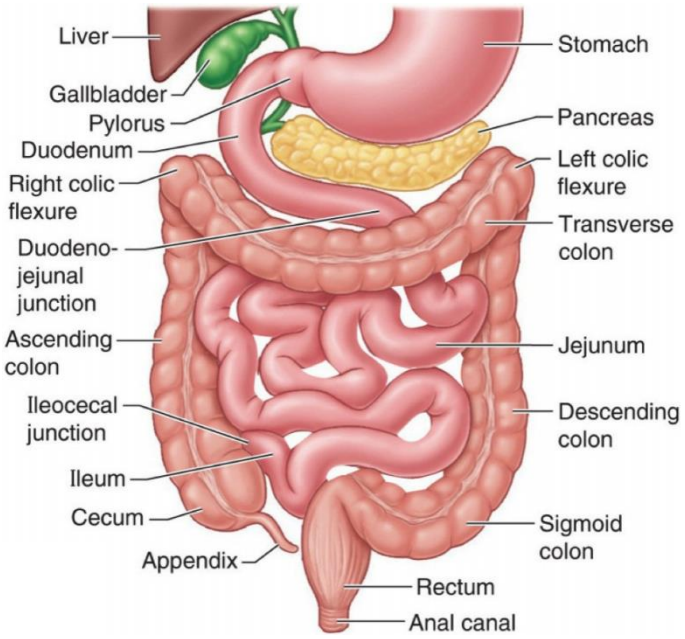

Figure 1. Part of the anatomy of the human GI tract [13]

Recently, there became a growing interest in studying the relation between gut microbiota and health. The link between the microbes in the human gut and the development of obesity, cardiovascular disease, and metabolic syndromes is becoming clearer [15].

Gut microbiota are able to transform various compounds [17]. These metabolic activities of the gut microorganisms have major consequences for the host, which can be both beneficial and harmful. Examples of beneficial compounds produced by bacteria in the human gut are several vitamins [18]. Examples of harmful compounds produced by bacteria present in the gut are several toxins and other potentially harmful compounds (e.g. ammonia, amines) [19]. The microbiota also have interactions with the innate and adaptive immune system [2]. There are links between the gut microbiota and the development of obesity, cardiovascular disease, and metabolic syndromes [15]. This is why the gut microbiota may not be underestimated and should be studied thoroughly.

1.2. Aryl hydrocarbon receptor

Gut microbiota are also able to produce compounds that activate certain receptors. One of these receptors is the aryl hydrocarbon receptor (AhR) [15]. It is present in the human body, and it recently gained more interest due to its role in human health [20]. The AhR is present in the cytoplasm of cells in its inactivated form. When AhR becomes activated by a ligand, it moves to the nucleus. In the nucleus it binds with the aryl hydrocarbon receptor nuclear translocator (ARNT), and forms the AhR-ARNT complex. This complex activates expression of a battery of genes containing specific DNA enhancer sequences called aryl hydrocarbon response elements (AhREs), which are often xenobiotic-metabolising enzymes. These enzymes are mainly involved in the activation and detoxification of carcinogenic polycyclic aromatic hydrocarbons (PAH's) [21].

AhR is also a crucial regulator in maintaining intraepithelial lymphocytes (IELs) numbers in both the intestine and the skin [22]. IELs are among others important in epithelial barrier organisation and in wound repair [23]. Furthermore, AhR modulates the differentiation of dendritic cells [2]. The receptor is also involved in the promoting of T_H17-cell differentiation and T_{reg}-cell differentiation [24]. The stimulation of AhR may also inhibit the development of autoimmune diseases (including Crohn's disease) and allergies (including allergic rhinitis) [25-27]. These are various reasons why ligands for AhR may be useful ingredients for functional foods, which are foods containing health-promoting additives [28]. A downside is that AhR possibly has a role in stimulating tumour initiation, promotion and progression [29]. The receptor is interesting to study, since little is known about the adverse and beneficial properties [30].

1.2.1. Ligands of the aryl hydrocarbon receptor

There are synthetic and naturally occurring ligands of AhR, of which several groups are depicted in table 1. The ligands for AhR do not have the same affinity to bind to the receptor [31].

Table 1. Ligands of the aryl hydrocarbon receptor

Origin	Ligand group
Synthetic (environmental contaminants)	Halogenated aromatic hydrocarbons (HAH's) including dioxins [32]
	Polycyclic aromatic hydrocarbons (PAH's) [32]
Natural	Several polyphenols [33]
	Several indole derivatives, including tryptophan derivatives [31, 34-36]
	The arachidonic acid metabolites lipoxin A4 and prostaglandin G [37]
	Tetrapyrroles (such as bilirubin) [38]
	Modified LDL's [39]
	Several dietary carotenoids [32, 33]

Several tryptophan metabolites and indole derivatives are ligands for the human AhR [31, 34-36]. Tryptophan can be degraded through several pathways, including the serotonin, indole, and kynurenine pathway [40]. The kynurenine pathway is the main metabolic route of tryptophan degradation [41]. The metabolites of its pathway are called kynurenines. Some of these kynurenines are AhR ligands. Examples are kynurenine, kynurenic acid, anthranilic acid and quinolinic acid [36, 42]. Part of the kynurenine pathway is displayed in figure 2.

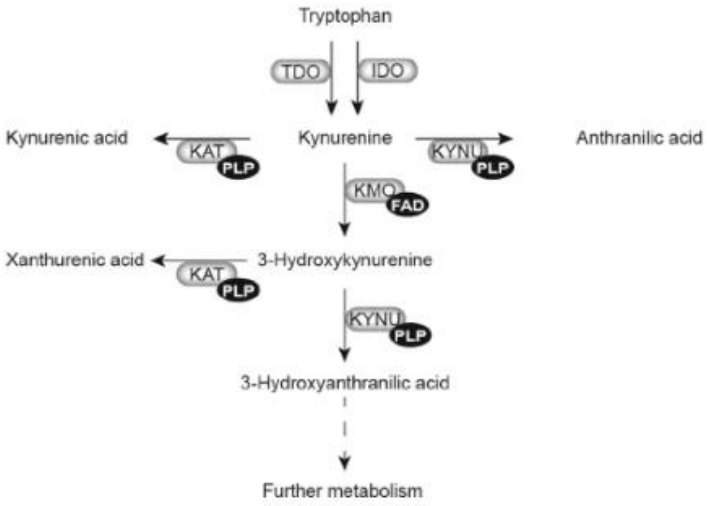

Figure 2. Part of the kynurenine pathway. The grey circles with letters inside are the enzymes responsible for the conversion [39].

Enzymes are needed for the conversion of tryptophan to kynurenine and other compounds. One of those enzymes, indoleamine 2,3-dioxygenase (IDO), is needed for the conversion of tryptophan to kynurenine. It was found that the presence of *Lactobacillus reuteri* increases the activity of this enzyme, which in turn increases the conversion of tryptophan to kynurenine [43, 44]. Lactobacilli (such as *Lactobacillus reuteri*) are the bacteria most involved in the production of tryptophan derivatives [45]. The *Lactobacillus reuteri* occurs naturally in humans, but not in all individuals [46].

The research by Park et al. (2015) shows that 6-formylindolo[3,2-b]carbazole (FICZ) is a tryptophan derivative and a very potent ligand for AhR [47]. This research also states that FICZ is formed when tryptophan is exposed to both visible and UV light.

In figure 3, the chemical structure of tryptophan and several tryptophan derivatives are shown, of which the compounds with a red dot are confirmed AhR ligands [45]. Like kynurenine, also xanthurenic acid and cinnabarinic acid are ligands of AhR generated by the IDO-enzyme. The research of Hubbard et al. (2015) suggests that 3-methyl indole, which is also called skatole, and 2-oxindole can stimulate the human aryl hydrocarbon receptor [48]. For many other tryptophan derivatives it is yet unsure whether those compounds are ligands.

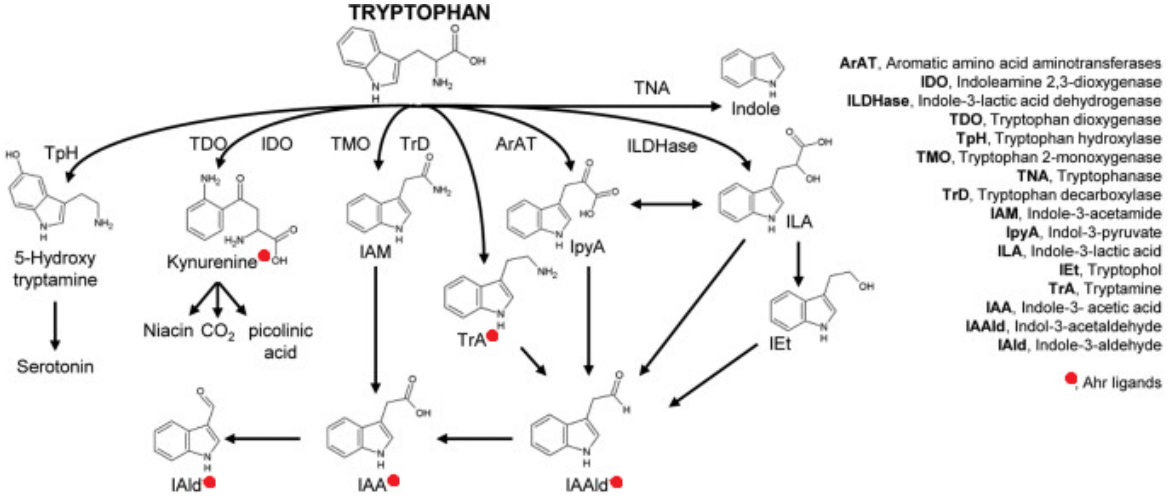

Figure 3. Tryptophan with several of its derivatives [43]

The compounds indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM), and indolo[3,2-b]carbazole (ICZ) are ligands for AhR as well [49-51]. I3C is an indole derivative. I3C is produced by the breakdown of the glucobrassicin, which is a glucosinolate [52]. I3C can be found in cruciferous vegetables such as broccoli, cabbage and Brussels sprouts [53, 54]. When I3C comes into contact with stomach acid, the condensation products DIM and ICZ are formed [55, 56]. ICZ may also be a derivative of tryptophan [57]. The affinity to bind AhR of these compounds is different. DIM, and especially I3C, have a relatively low affinity for AhR, while ICZ has one of the highest AhR binding affinities [51, 58-60]. The structural formulas of I3C, DIM, and ICZ are depicted in figure 4 [61].

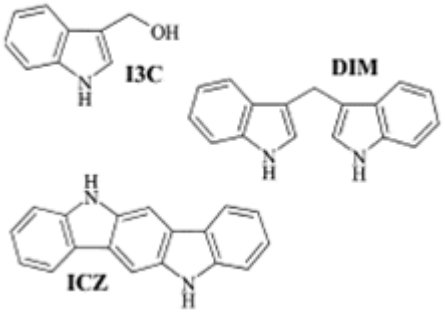

Figure 4. Chemical structure of I3C, DIM, and ICZ [58]

1.3. Research objective and hypothesis

Previous research showed that the bacterial supernatant originating from SHIME® fermentation gives a high AhR activation (approximately 40% of the positive control β-naphthoflavone). This supernatant consists of both SHIME® growth medium and bacterial metabolites.

Because of the high AhR activation in the bacterial supernatant, this study aimed to identify the compounds that trigger AhR and are present in the microbial supernatant originating from SHIME® fermentation. This gives the following research question:

- *Which compound(s) that trigger AhR are present in the microbial supernatant originating from SHIME® fermentation?*

Suspected is that the compounds are derivatives of the amino acid tryptophan. There are proteins present in the growth medium, specifically in yeast extract, special peptone and mucin [62]. These proteins consist of amino acid, which includes tryptophan. So tryptophan is present in the nutrients for the bacteria. When there is an unrestricted availability of tryptophan, several bacteria strains are able to metabolise tryptophan to derivatives that can trigger AhR [45].

2. Materials and methods

2.1. Chemicals

Purified water was used unless stated otherwise. Ultrapure water was made by the PURELAB® Ultra from Elga.

Chemicals used for HPLC analysis

ULC-MS-grade acetonitrile and HPLC-grade methanol were purchased from Actu-All Chemicals. Puriss. p.a. formic acid (formic acid:water 1:1) for HPLC was purchased from Sigma-Aldrich.

99% DL-tryptophan, indole, L-kynurenine, 5,11-dihydroindolo[3,2-b]carbazole-6-carbaldehyde (FICZ), indole-3-carbinol (I3C), and 3,3'-diindolylmethane (DIM) were all purchased from Sigma-Aldrich. DL-Tryptophan was tested at concentrations of 24µM (5µg/mL), 49µM (10µg/mL), 122µM (25µg/mL), 245µM (50µg/mL), 734µM (150µg/mL), and 1224µM (250µg/mL). Indole was tested at concentrations of 43µM (5µg/mL), 213µM (25µg/mL), 426µM (50µg/mL), and 1280µM (150µg/mL). L-Kynurenine was tested at concentrations of 24µM (5µg/mL), 48µM (10µg/mL), 120µM (25µg/mL), 240µM (50µg/mL), 720µM (150µg/mL), and 1201µM (250µg/mL). FICZ was tested at a concentration of 100µM (28.4µg/mL). I3C was tested at the following concentrations: 34µM (5µg/mL), 170µM (25µg/mL), 340µM (50µg/mL), 510µM (75µg/mL), 679µM (100µg/mL), and 1019µM (150µg/mL). DIM was tested at the following concentrations: 20µM (5µg/mL), 101µM (25µg/mL), 203µM (50µg/mL), 304µM (75µg/mL), 406µM (100µg/mL), and 609µM (150µg/mL).

Chemicals used for fractionation

PEC-grade hexane was purchased from Actu-All Chemicals. Ethyl acetate for analysis was produced by EMSURE®.

Chemicals used for CALUX assay

Dimethylsulphoxide (DMSO, analytical grade) was purchased from Merck KgaA. Lysis buffer and luciferase assay buffer were purchased from Promega. Alpha MEM Gibco™ GlutaMAX™ and Northern American heat inactivated foetal calf serum (FCS) from Gibco® were purchased from ThermoFisher Scientific. β-naphthoflavone and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich.

99% DL-tryptophan, indole, L-kynurenine, and indole-3-carbinol (I3C) were all purchased from Sigma-Aldrich. DL-Tryptophan and indole were tested at the following concentrations: 10µM, 20µM, and 40µM. L-Kynurenine was tested at a concentration of 40µM, and I3C was tested at a concentration of 20µM.

Chemicals used for growing bacteria

YCFA medium was made according to the article written by Duncan et al., with classified modifications [63]. SHIME® medium was purchased from ProDigest. The bacterial growth medium used for the L-SHIME® contained 1.2g/L arabinogalactan, 2g/L pectin, 0.5g/L xylan, 0.4g/L glucose, 3g/L yeast extract, 1g/L special peptone, 3g/L mucin, 0.5g/L L-cysteine-HCl and 4g/L starch [62]. The only difference in the composition of the bacterial growth medium for the M- SHIME® was the concentration of mucin (2g/L instead of 3g/L).

2.2. SHIME®

The role of the microbiota in the gut was investigated by using the Simulator of Human Intestinal Microbial Ecosystem (SHIME®). The SHIME® mimics the human gastrointestinal tract, so it is an *in vitro* fermentation system. Faeces of human donors supply the inoculum bacteria and a stabilisation

period of two weeks with pH control ensures a stable microbiota before the experiment. Standardised feed was used [62, 64]. The colonic bacteria convert the nutrients into metabolites.

SHIME®-samples originated from an M(ucin)-TWINSHIME® model, where the model includes mucin beads, with stable microbiota from one donor. Two biological replicates were used. In the TWINSHIME® model, the colon is divided in three parts: the ascending colon (pH=5.6-5.9), the transverse colon (pH=6.15-6.4), and the descending colon (pH=6.6-6.9) [62]. Also L(uminal)-SHIME® samples (without mucin beads) were used. These originated from a TRIPLESHIME® model with stable microbiota from three donors. These samples were used for comparison with the M-TWINSHIME® model. In the TRIPLESHIME® model, the colon is divided in two parts: the proximal colon (pH=5.6-5.9) and the distal colon (pH=6.6-6.9) [62]. This means that the distal colon has the same pH range as the descending colon. Only distal colon supernatant was used out of the L-SHIME® system, so no proximal colon supernatant was used.

The SHIME®-samples were first centrifuged for 5 minutes at 9000*g* and 4°C. The supernatant was filtrated through a 0.2µM RC filter and stored in -20°C until further analysis. The filtrated supernatant was used in the experiments.

2.3. Fractionation with solvents

The descending colon supernatant, originating from M-SHIME® fermentation, was fractionated based on polarity. This was done by mixing (shaking and vortexing) 5mL of the filtrated supernatant in 5mL solvent. The solvents used were hexane, as a non-polar solvent, and ethyl acetate, as a polar aprotic solvent. After mixing, the organic layers were separated into different tubes. This procedure was repeated, so again 5mL of organic solvent was mixed with the same supernatant. After this, the organic layers were again separated and combined with the first fractionated organic layers. The watery layers were also separated and put into different tubes. The organic fractions were dried using an N_2 flow. After the drying step the dry matter was redissolved in 4x less solvents, resulting in a 4x more concentrated fraction.

2.4. HPLC method

High-performance liquid chromatography (HPLC) was used to detect several compounds and to capture peaks (as discussed in 2.6). The column used was the Varian Polaris 5 C18-A 4.6 x 150mm, with the column oven set to 30°C. Two eluents were used: eluent A contained ultrapure water with 0.05% formic acid, and eluent B contained acetonitrile with 0.05% formic acid. The eluents were sonicated for 20 minutes. These eluents were degassed via an online degasser. The injection volume was set at 20µL. Each run lasted 31 minutes. At the start of the run, the gradient was A:B=100:0. The gradient increased linearly to A:B=58:42, after which the gradient remained the same for 5 minutes. Between minute 25 and 26 the gradient was linearly decreased to A:B=100:0, which remained till the end of the run. The flow was 1mL/min. The peaks were detected with a UV visible detector, with the wavelengths set at 210nm, 225nm, 280nm and 520nm.

The pure compounds tested (DL-tryptophan, indole, L-kynurenine, FICZ, I3C, and DIM) were dissolved in 100% methanol.

2.5. Freeze-drying

The water-fraction obtained by mixing hexane and the descending colon supernatant, originating from M-SHIME® fermentation, has been freeze-dried till no fluid was present anymore. The remaining dry matter has been dissolved again in water to a concentration 30x higher than before freeze-drying. The concentrated water-fraction of the descending colon supernatant has been fractionated using HPLC, as described in paragraph 2.6.

2.6. Fractionation with HPLC

The (concentrated water-fraction of the) descending colon supernatant, originating from M-SHIME® fermentation, were fractionated using HPLC (method in 2.4). When a peak eluted, the fluid was captured. The time between the detection of the peaks and the capturing was calculated. The internal diameter of the tube between the UV visible detector and the waste bin was equal to 0.13mm and the length of this tube was equal to 50cm. This gave an internal volume of 0.0066mL, so with the flow of 1mL/min this meant that the time between the detection of the peaks and the capturing was 0.4 seconds, so negligible. Unlike samples that were tested with HPLC, the injection volume of samples fractionated with HPLC was set at 30µL.

The AhR activation of the captured peaks was tested on the CALUX® assay as described in paragraph 2.7.

2.7. CALUX® bioassay

The AhR activation was measured in technical triplicates using the Chemical Activated LUciferase gene eXpression (CALUX®) assay (HMI, Wageningen University). The activation is expressed as the percentage activation of β-naphthoflavone (5µM), which is the positive control of this assay. β-naphthoflavone is a strong AhR agonist [65, 66].

The CALUX® cells were split twice a week, on Monday and Friday. These cells were grown in A-MEM + 1% penicillin/streptomycin and 10% heat inactivated foetal calf serum (FCS).

For the seeding of the CALUX® cells, a confluent T75 flask (trypsin-EDTA) was trypsinised. The cells were counted and diluted to $7.5*10^5$ cells/mL. 100µL of this concentration was seeded per well to reach a final concentration of $7.5*14^4$ cells/mL for each well. After seeding, the 96-wells plate (Corning®, polystyrene, white plate, white flat bottom) was incubated for 24 hours at 37°C, before stimulation. After these 24 hours, the cell layer was confluent. Following up to these 24 hours, the cells were again incubated for 24 hours, together with the stimuli. After these 24 hours the assay was performed.

For the assay, the buffers (lysis buffer, luciferase assay buffer and PBS) were thawed and pre-warmed in a water bath to 37°C. After pre-warming, the pH of the lysis buffer was set to 7.8. The cells, present in the wells, were washed twice with 200µL of pre-warmed PBS. After the washing, the cells were lysed with 20µL lysis buffer/well. With this lysis buffer, the plate was carefully stirred for 10 minutes at room temperature, whereafter 100µL assay buffer was added to each well. The luminescence was measured immediately with the SpectraMax® M5 (Molecular devices).

Samples with an activation higher than 5% of the activation of β-naphthoflavone were considered to activate AhR.

2.8. Microbiota that metabolize tryptophan

Three strains of bacteria (confidential), that were according to literature able to break down tryptophan, were grown on both L-SHIME® medium and a control medium (YCFA) and were diluted to an optical density (OD600) of 0.1 (HMI, Wageningen University). The samples were centrifuged at 4000*g* for 20 minutes and the supernatant was filtered using a 0.2µM RC filter. This supernatant was stored at -80°C.

A CALUX® assay was performed with the bacteria and their medium. The bacteria and their medium were also tested using the HPLC.

2.9. Statistical analysis

Results are expressed as mean values ± standard error of the mean. All the data was analysed by unpaired t-tests using IBM SPSS Statistics 23. Statistical significance was set at $P < 0.05$. $P < 0.05$ was indicated with *, $P < 0.01$ was indicated with **. Statistical differences between treatments ($P < 0.05$) were indicated with a, b, c, and d.

3. Results

3.1. AhR activation by colon metabolites

The AhR activation of different colon parts is shown in figure 5. The ascending colon (AC) supernatant, transverse colon (TC) supernatant, and the descending colon (DC) supernatant, originating from M-SHIME® fermentation were tested. These three supernatants all had an AhR activation significantly higher than the negative control ($P < 0.01$). The AC supernatant (22.745% ± 0.683), TC supernatant (26.078% ± 2.039), and DC supernatant (34.279% ± 1.502) also had an AhR activation significantly above 5% of the activation of β-naphthoflavone ($P < 0.05$), which indicates that the three supernatants, originating from different parts of the colon, all activated AhR. It seems like the AhR activation increased from AC to TC to DC. The AhR activation of the descending colon was significantly higher than both the AC and the TC ($P < 0.05$). However, the activation by the TC supernatant was not significantly higher than the activation by the AC supernatant ($P > 0.05$).

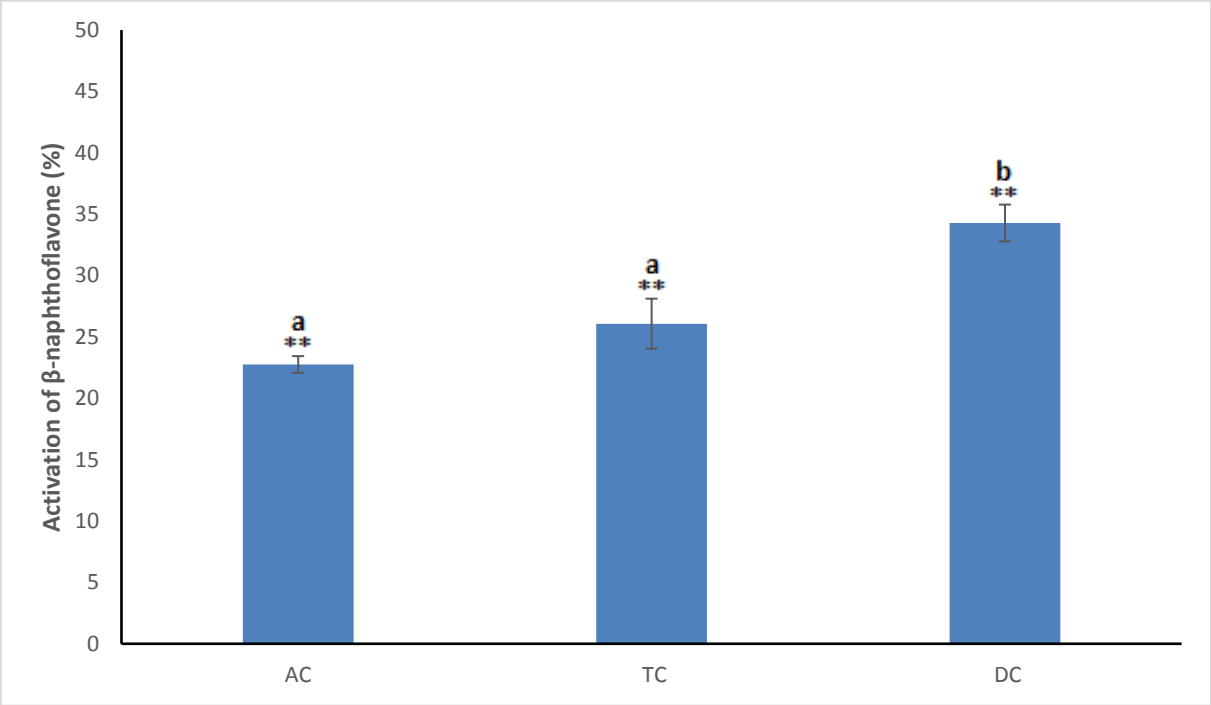

Figure 5. AhR activation of the supernatant of several parts of the colon, originating from M-SHIME® fermentation (10µL/well), determined by the CALUX® assay. AC=ascending colon, TC=transverse colon, and DC=descending colon

3.2. HPLC chromatograms of different colon parts and tryptophan

As seen in figure 6, differences in composition are found between M-SHIME® samples from the ascending colon, the transverse colon and the descending colon. Three peaks in the AC, TC, and DC, were considerably larger than other peaks. The very first peak that immediately went below zero, is not taken into account since it was most probably due to the eluent. As shown in figure 6, the three peaks are indicated with “peak A”, “peak B”, and “peak C”. Peak A eluted from minute 3.7 till minute 4.1, peak B eluted from minute 14.3 till minute 15.0 minutes, and peak C eluted from minute 20.1 till minute 20.9.

Tryptophan has also been measured on the HPLC, as seen in figure 7. It eluted between 6.6 minutes and 7.8 minutes. When comparing this chromatogram to the chromatograms of the M-SHIME® samples (figure 6), tryptophan is found to be present in the ascending, transverse, and descending

colon. When looking at the chromatograms in figure 6, the peaks seem to decrease in size from AC to TC to DC. When calculating the concentrations with the calibration curve of tryptophan (figure 22, Appendix II), the concentration was found to go from 321µM in the ascending colon, to 41µM in the transverse colon, and to 19µM in the descending colon.

Like the peak of tryptophan, peak A also seemed to decrease. The area of peak A went from 81mAU*min in the ascending colon, to 75mAU*min in the transverse colon, to 69mAU*min in the descending colon. Peak B and peak C seemed to increase. The area of peak B went from 50mAU*min in the ascending colon, to 91mAU*min in the transverse colon, and to 123mAU*min in the descending colon. Peak C went from 34mAU*min in the ascending colon, to 78mAU*min in the transverse colon, and to 94mAU*min in the descending colon.

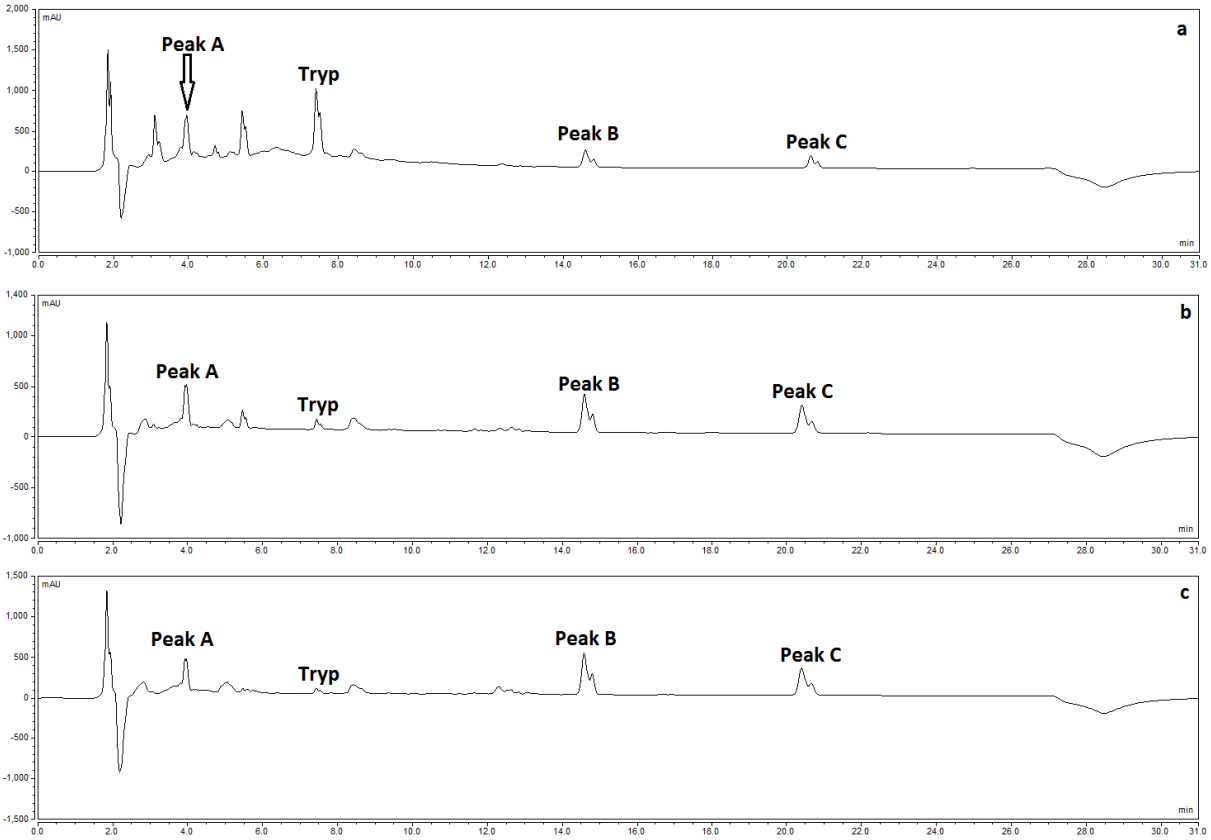

Figure 6. HPLC chromatograms (210nm) of M-SHIME® samples
a: Ascending colon
b: Transverse colon
c: Descending colon
Tryp: Tryptophan

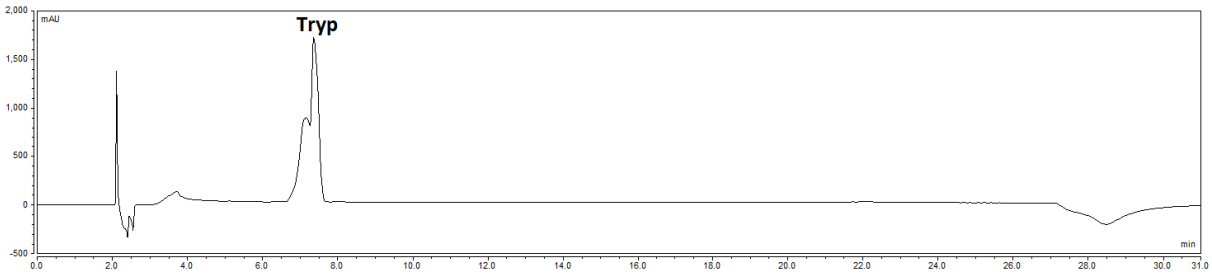

Figure 7. HPLC chromatogram (210 nm) of 1224µM DL-tryptophan (Tryp)

3.3. Differences between M-SHIME® fermentation and L-SHIME® fermentation

When comparing the chromatograms of the descending colon supernatant, originating from M-SHIME® fermentation (figure 6c), with the distal colon supernatant, originating from L-SHIME® fermentation (figure 8), it can be seen that peak A was not clearly present in the distal colon supernatant. The UV-VIS spectrum of peak A was not found in the chromatogram of the distal colon supernatant. Peak B and peak C were present, but in lower concentrations than in the descending colon supernatant. In the descending colon supernatant, peak B and peak C had an area of respectively 123mAU*min and 94mAU*min. The area of peak B and peak C differed for different donors. The areas of peak B from the three donors were respectively 45mAU*min, 21mAU*min, and 43mAU*min. The areas of peak C from the three donors were respectively 36mAU*min, 29mAU*min, and 28mAU*min.

There are more differences between the chromatograms of M-SHIME® fermentation and L-SHIME® fermentation, especially between 2.5 minutes and 6 minutes. There is also a peak after peak C in the chromatograms of L-SHIME® fermentation, that is not present in the chromatograms of M-SHIME® fermentation, eluting from 21.9 minutes till 22.7 minutes.

There are also differences between the chromatograms of different donors in L-SHIME® fermentation. As said before, there are differences between the areas of peak B and peak C. Also between 2.5 minutes and 6 minutes there are differences between the chromatograms (figure 8).

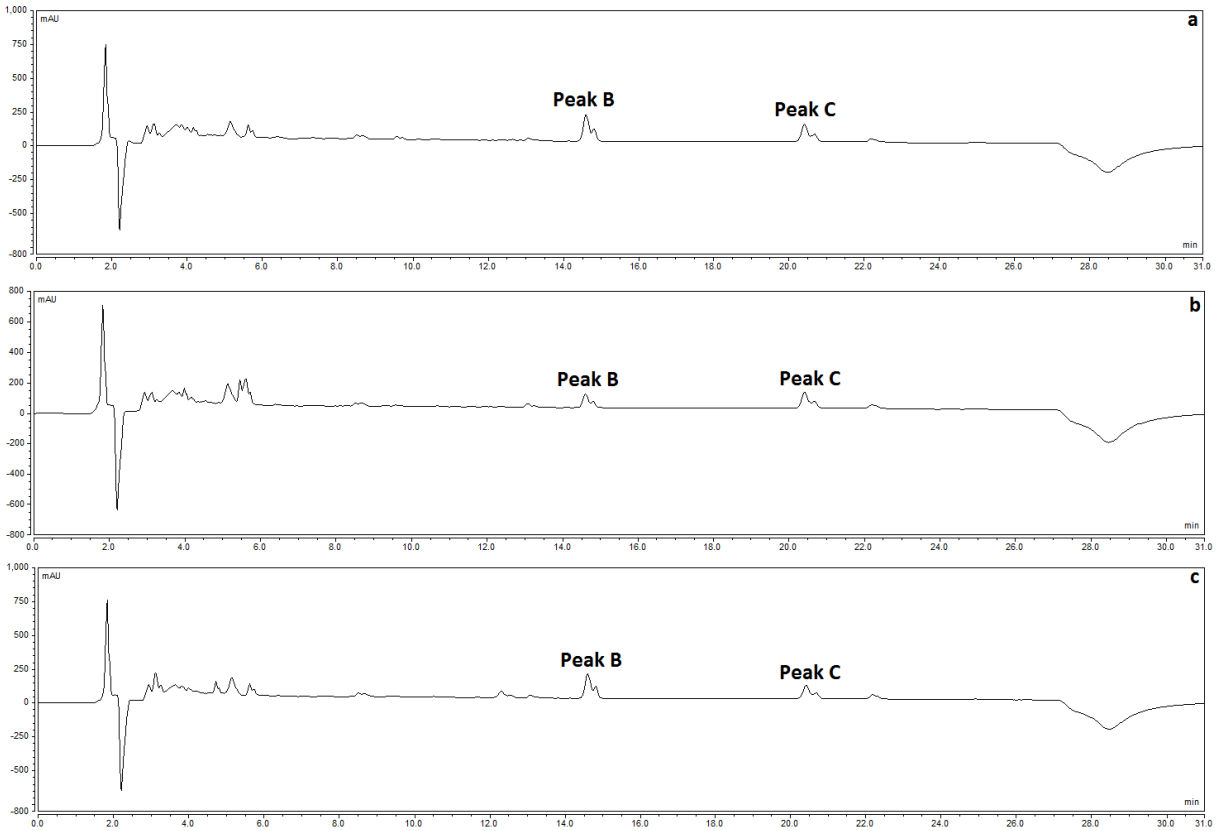

Figure 8. HPLC chromatogram (210nm) of the distal colon supernatant originating from L- SHIME® fermentation
a: Donor 1
b: Donor 2
c: Donor 3

3.4. AhR activation of fractionated M-SHIME® samples

The descending colon supernatant originating from M-SHIME® fermentation was fractionated with ethyl acetate and hexane. The descending colon supernatant was chosen because this had the highest AhR activation. In figure 9 the AhR activations of the four fractions (water from hexane, hexane, water from ethyl acetate, and ethyl acetate) can be seen. The activation of the hexane fraction was not significantly different compared to the negative control ($P > 0.05$). The other three fractions had an activation significantly higher than the negative control ($P < 0.01$). However, it must be noted that the activation of the ethyl acetate fraction is below 5% of the activation of β-naphthoflavone (0.538% ± 0.084). Both water fractions of the descending colon supernatant had an AhR activation above 5% of the activation of β-naphthoflavone. The water fraction of the descending colon supernatant, obtained by mixing the DC supernatant with hexane, was found to have a higher activation than the non-fractionated descending colon ($P < 0.05$). The AhR activation of the water fraction was 46.516% ± 2.435, while the AhR activation of the non-fractionated descending colon supernatant was 34.279% ± 1.502.

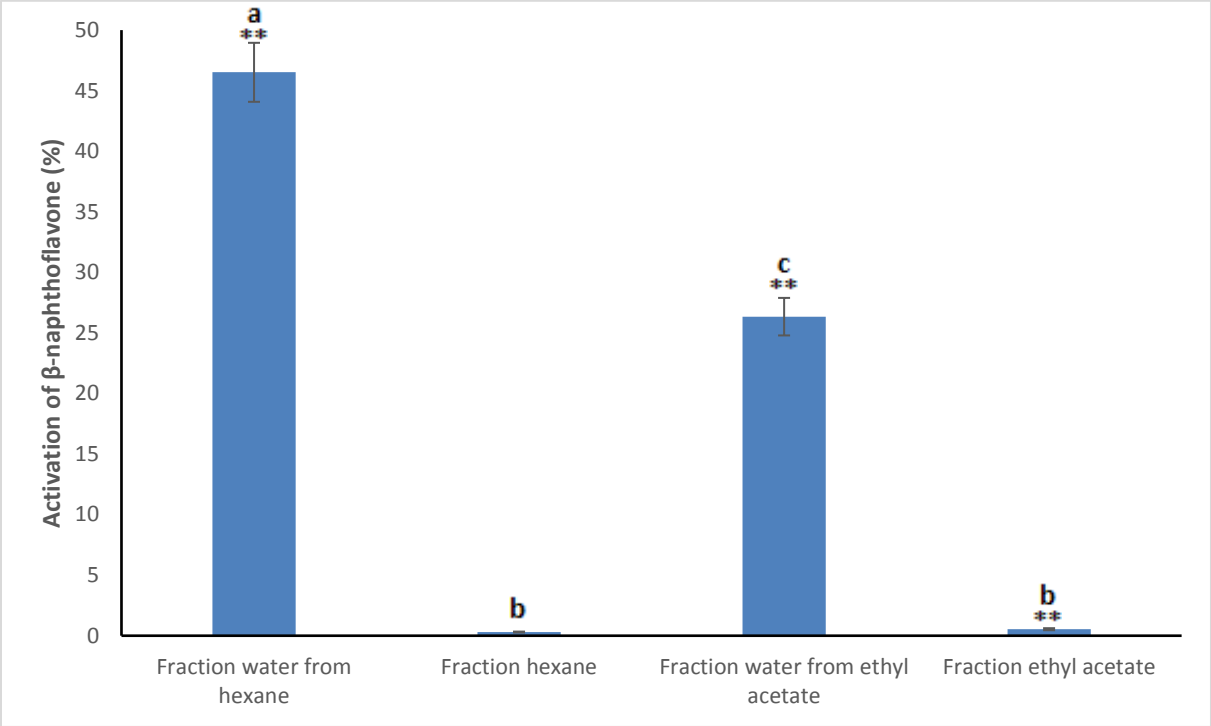

Figure 9. AhR activation of the descending colon supernatant (10µL/well), originating from M-SHIME® fermentation, that was fractionated using hexane and ethyl acetate

Peak B was captured from the concentrated descending colon supernatant, obtained by M-SHIME® fermentation. Different amounts of peak B were tested (1µL, 10µL, 15µL) on the CALUX® assay. The AhR activations compared to the AhR activation of β-naphthoflavone were respectively 0.487% ± 0.260, 0.125% ± 0.140, and 0.547% ± 0.130 (figure 10). The three different concentrations were not significantly different from each other ($P > 0.05$). The two lowest concentrations (1µL and 10µL) were not significantly different from the negative control ($P > 0.05$), but the highest concentration (15µL) was significantly higher than the negative control ($P < 0.05$). However, this value is still relatively low, below 5% of the AhR activation of β-naphthoflavone.

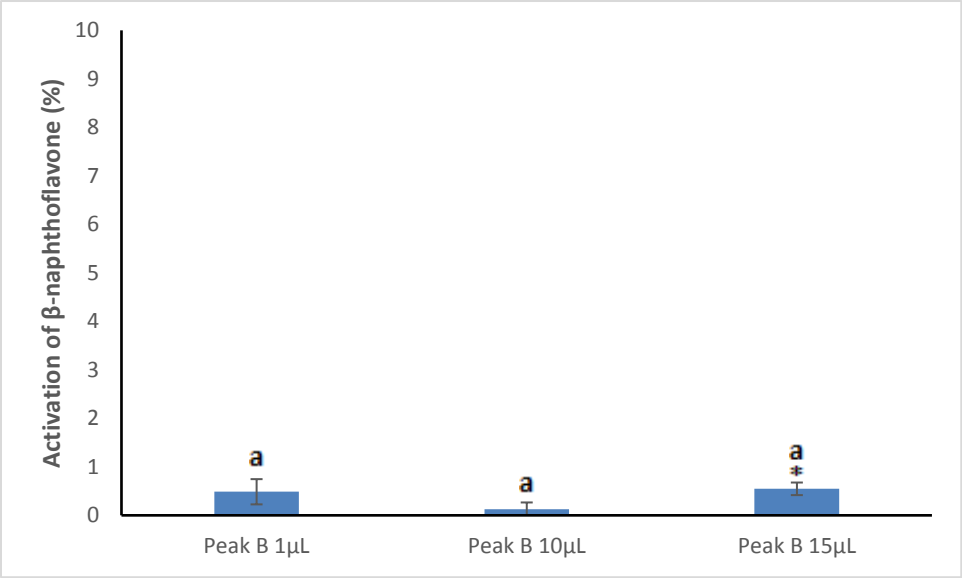

Figure 10. AhR activation of different concentrations of the captured peak B

3.5. HPLC chromatograms of fractionated M-SHIME® samples

Peak A and peak B were found to be soluble in the water-fraction. Peak C was found to be soluble in the organic-fractions (hexane and ethyl acetate). The HPLC chromatogram of the water-fraction, obtained by mixing hexane with the descending colon supernatant, originating from M-SHIME® fermentation, is depicted in figure 11. The hexane-fraction, obtained the same way, is also depicted in figure 11. Two other fractions, obtained by mixing the descending colon supernatant originating from M-SHIME® fermentation, with ethyl acetate, are depicted in figure 12. These chromatograms are comparable to those in figure 11, with the biggest difference that ethyl acetate also elutes at a retention time ranging from 5.0 till 9.7 minutes in the water-fraction, and ranging from 7.9 till 9.2 minutes in the ethyl acetate-fraction (figure 20, Appendix I).

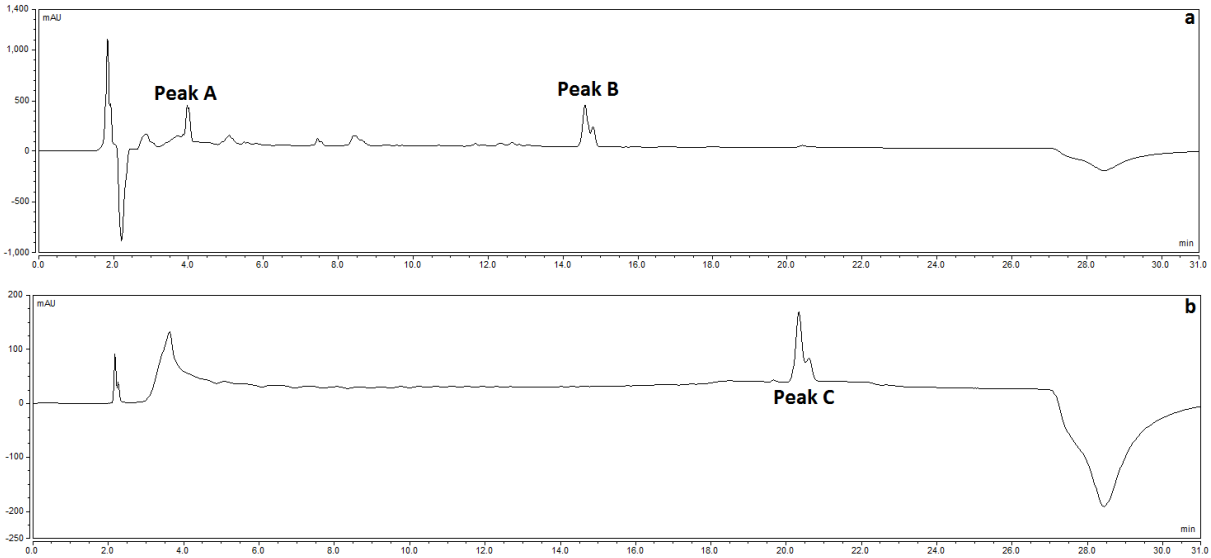

Figure 11. HPLC chromatogram (210nm) of the fractionated descending colon supernatant, obtained by M-SHIME® fermentation
a: Water-fraction obtained from mixing the descending colon supernatant with hexane
b: Hexane-fraction obtained from mixing the descending colon supernatant with hexane

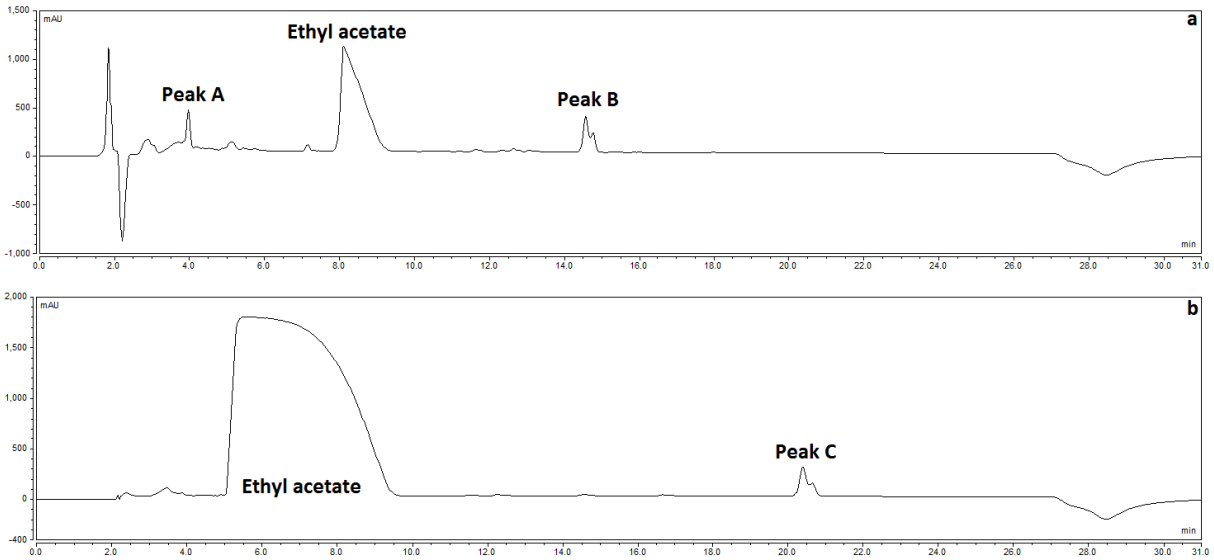

Figure 12. HPLC chromatogram (210nm) of the fractionated descending colon supernatant, obtained by M-SHIME® fermentation
a: Water-fraction obtained from mixing the descending colon supernatant with ethyl acetate
b: Ethyl acetate-fraction obtained from mixing the descending colon supernatant with ethyl acetate

3.6. AhR activation of DL-tryptophan, indole, L-kynurenine, and I3C

All different concentrations of DL-tryptophan tested were significantly higher than the negative control ($P < 0.05$, figure 13). For an increasing concentration of 10µM, 20µM, and 40µM, the AhR activation increased respectively from 1.354% ± 0.250, 2.326% ± 0.117, and 5.239% ± 0.152. The activation showed a dose dependent response ($P < 0.05$). For a concentration of 40µM DL-tryptophan, the AhR activation was higher than 5% of the activation of β-naphthoflavone, though not significantly higher ($P > 0.05$).

There was no significant difference between AhR activation of different concentrations of indole ($P > 0.05$, figure 14). The AhR activations of 20µM and 40µM indole were also not significantly higher than the negative control ($P > 0.05$), but the activation of 10µM indole was ($P < 0.01$). For an increasing concentration of 10µM, 20µM, and 40µM, the corresponding AhR activations found were 0.352% ± 0.036, 0.207% ± 0.085, and 0.675% ± 0.184.

The AhR activation of 40µM of L-kynurenine was equal to 1.066% ± 0.154 (figure 15). This value was significantly higher than the negative control ($P < 0.01$). However, this value is still relatively low, below 5% of the AhR activation of β-naphthoflavone.

The AhR activation of 20µM of I3C was found to be 1.185% ± 0.104, which was significantly higher than the negative control ($P < 0.05$, figure 16). However, here it must also be noted that the activation is relatively low, below 5% of the activation of β-naphthoflavone.

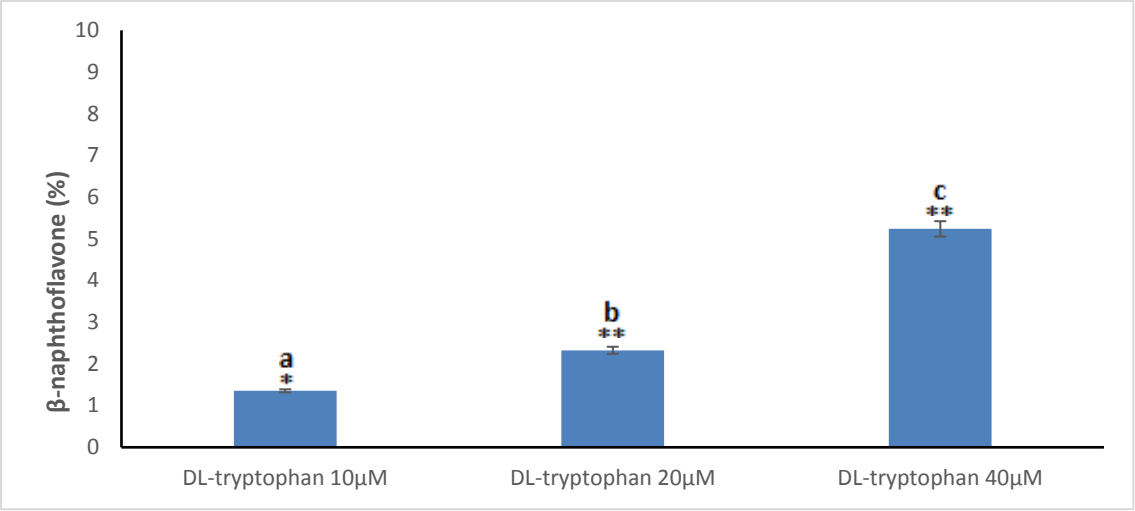

Figure 13. AhR activation of different concentrations of DL-tryptophan

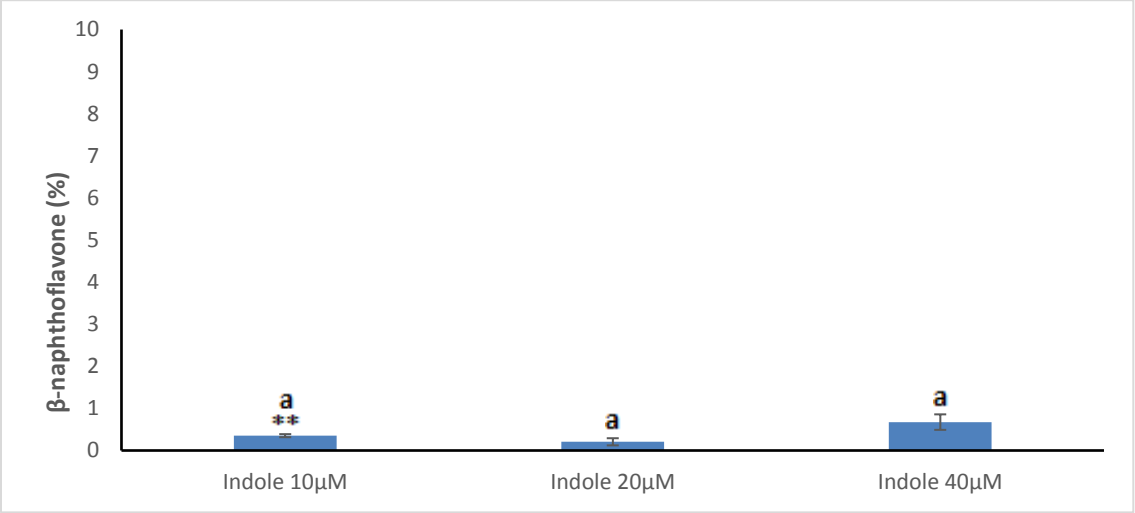

Figure 14. AhR activation of different concentrations of indole

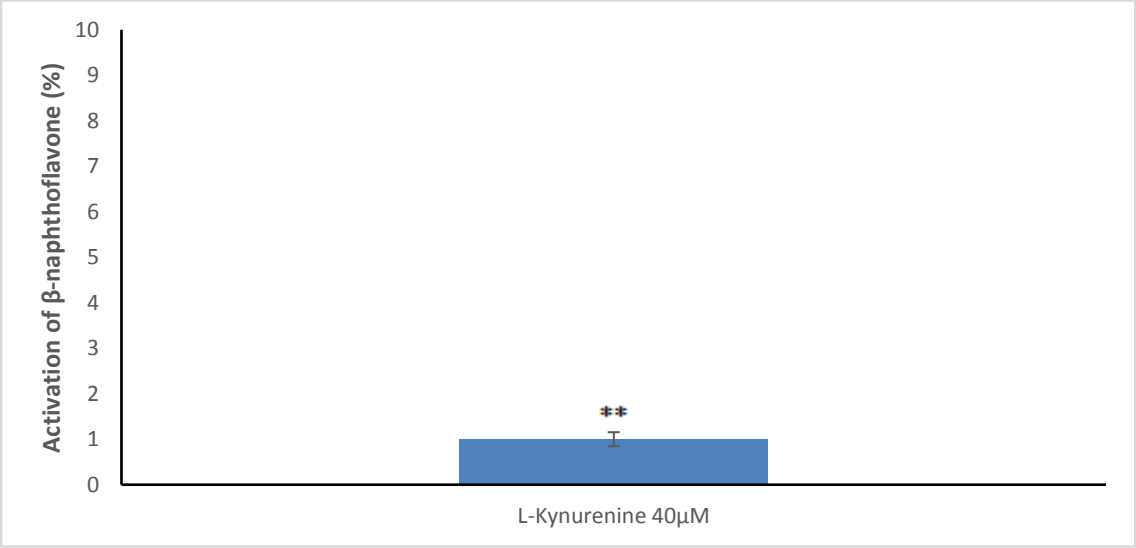

Figure 15. AhR activation of 40µM L-kynurenine

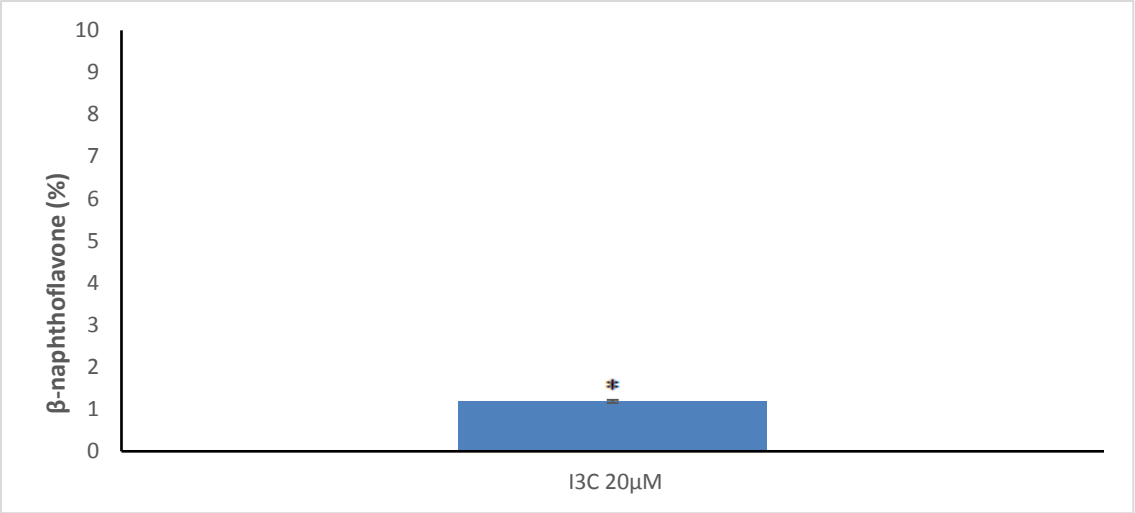

Figure 16. AhR activation of 20µM indole-3-carbinol (I3C)

3.7. HPLC chromatograms of indole, L-kynurenine, I3C, DIM, and FICZ

Indole, L-kynurenine, FICZ, I3C, and DIM were measured on the HPLC (figure 17). Indole was found in the supernatants of all three different colon parts, originating from M-SHIME® fermentation (figure 6). Indole is the same compound as peak C, since peak C and indole had the same retention times (20.1 till 20.9 minutes). The UV-VIS spectra were also the same. Since peak C was identified as indole, the concentrations in the M-SHIME® samples could be calculated. The calibration curve can be found in figure 23 (Appendix II). The concentration of indole in the ascending colon, transverse colon and descending colon were respectively 79µM, 182µM, and 218µM. I3C, DIM, and FICZ were not identified in the different M-SHIME® supernatants.

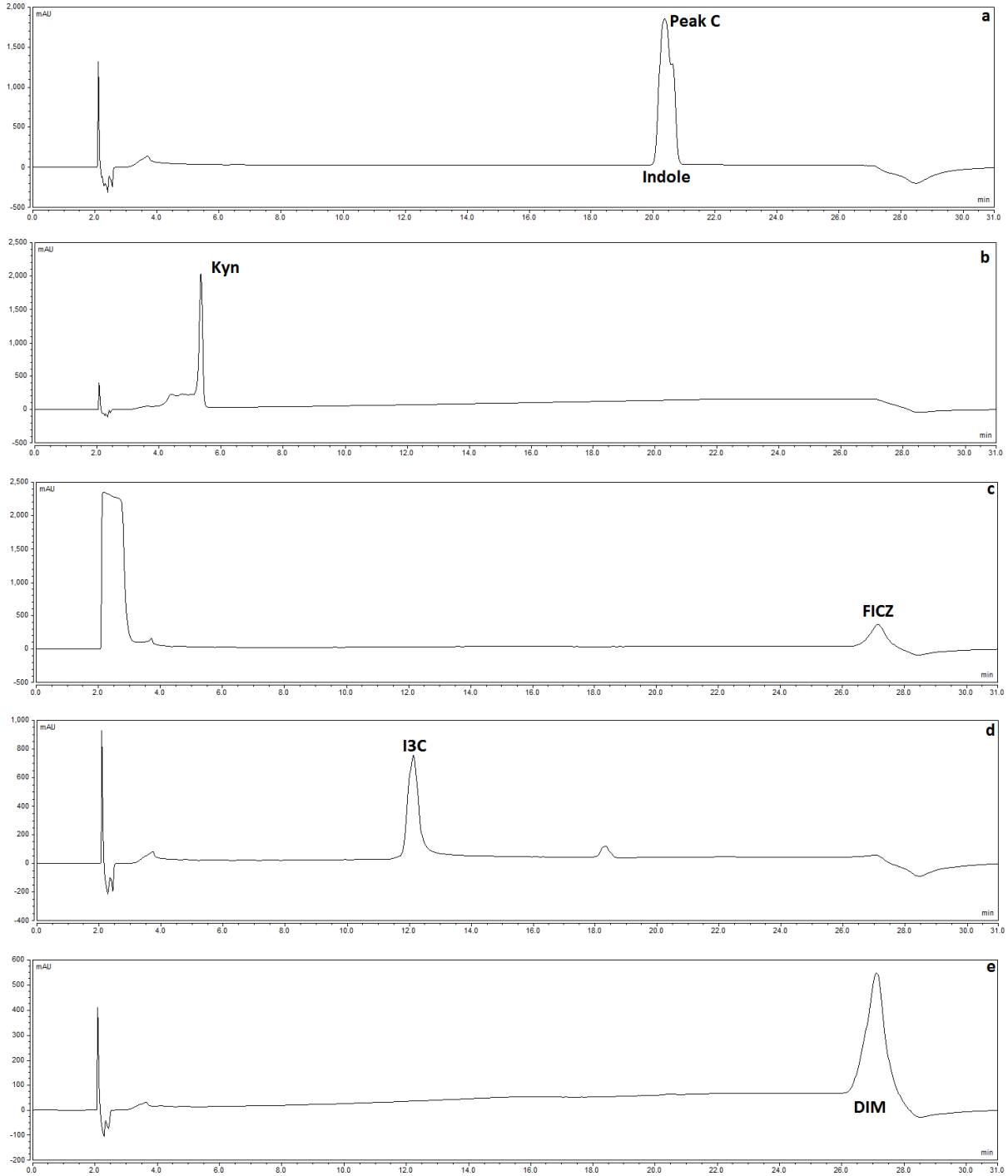

Figure 17. HPLC chromatograms of various pure compounds (210nm for A, C and D, 225nm for B and E)
a: Indole (1280µM)
b: L-Kynurenine (Kyn, 1201µM)
c: FICZ (100µM)
d: I3C (1019µM)
e: DIM (609µM)

3.8. Microbiota that metabolise tryptophan

The three bacterial strains in both SHIME® medium and YCFA medium had a lower activation than the medium without bacteria ($P < 0.05$, figure 18). Strain B in YCFA medium was not found to have a significantly higher AhR activation than the negative control ($P > 0.05$). The media (YCFA medium and SHIME® medium) and the other bacteria strains (strain A in both YCFA medium and SHIME® medium, strain B in SHIME® medium, and strain C in both YCFA medium and SHIME® medium) were found to have a significantly higher AhR activation than the negative control ($P < 0.05$). However, it must be noted that the activations were still relatively low, especially the bacteria strains. There were no significant differences in activation for strain B in different media ($P > 0.05$), and for strain C in different media ($P > 0.05$). There was a significant difference for strain A, so the AhR activation of strain A in the SHIME® medium is higher than the activation of strain A in YCFA medium ($P < 0.05$).

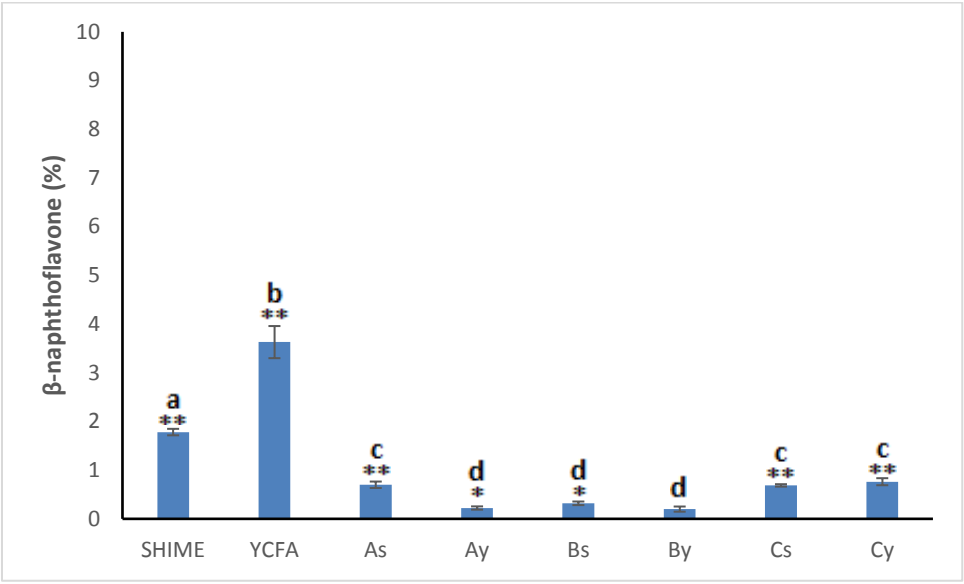

Figure 18. AhR activation of three strains of bacteria (A, B, C) in both SHIME® medium (s) and YCFA medium (y)
As, Bs, Cs: strain A, B, C in SHIME® medium
Ay, By, Cy: strain A, B, C in YCFA medium

In figure 19 the HPLC chromatograms of SHIME® medium and the three bacteria strains that were grown in SHIME® medium are depicted. These four chromatograms did not have any big differences. The only notable difference is the peak eluting at 4.5 minutes. This peak is higher in the medium and strain C (respectively 768mAU and 669mAU) than in strain A and strain B (respectively 372mAU and 338mAU). Tryptophan is also identified in these chromatograms. There was no clear difference in concentration between the peaks of tryptophan in the four different chromatograms. The concentration for SHIME® medium, and strain A, B, and C in SHIME® medium, are respectively 149µM, 139µM, 155µM, and 128µM. The same story holds true for the HPLC chromatograms of YCFA medium, and the three strains in YCFA medium. These four chromatograms are depicted in figure 21 (Appendix I).

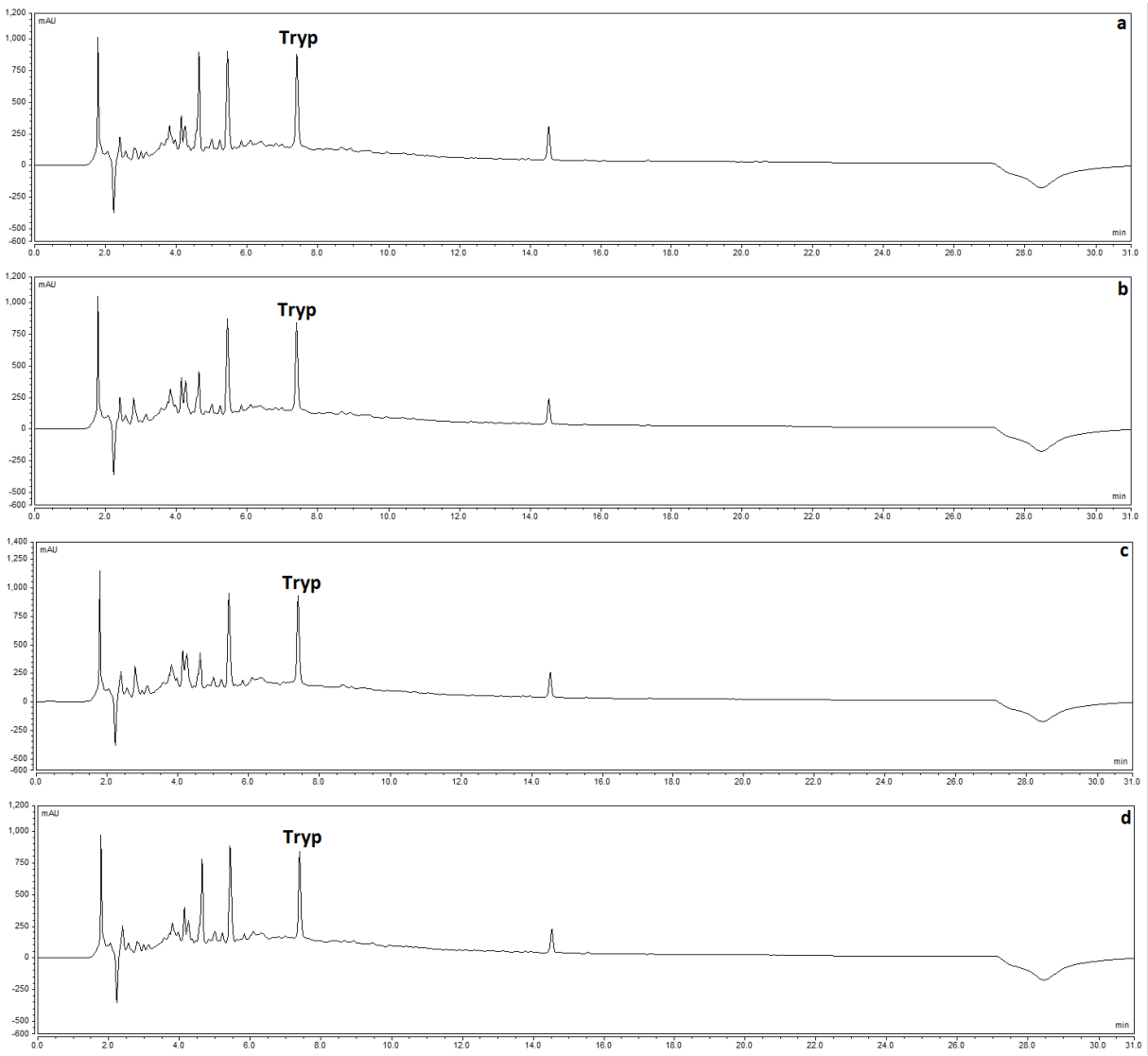

Figure 19. HPLC chromatograms (210nm)
a: SHIME® medium
b: Bacteria strain 1 growing in SHIME® medium
c: Bacteria strain 2 growing in SHIME® medium
d: Bacteria strain 3 growing in SHIME® medium
Tryp: tryptophan

4. Discussion

4.1. AhR activating compounds in bacterial supernatant originating from the M-SHIME®

The AhR activation was found to be the highest in the descending colon, followed by the transverse colon and the ascending colon. This indicates that the bacteria present in the descending colon metabolise certain molecules to AhR ligands. The amount of tryptophan present decreases from the ascending colon to the transverse colon and the descending colon, so the bacteria present in the colon seem to metabolise tryptophan.

It must be noted that the SHIME® system does not include absorption, which is a limitation of the SHIME® system. In this research there seemed to be more activation in the descending colon, but in real humans this is maybe not the case, since the compounds are absorbed. Part of the tryptophan present is probably also absorbed before it gets metabolised by gut microbiota. To our best knowledge, no study is done with different sections of the colon and the tryptophan breakdown capacity. The same is the case with different sections of the colon and the amount of AhR ligands present. The results in our study show a possible causality between these two, there seems to be more AhR activation with a decreasing concentration of tryptophan present. This could mean that tryptophan is metabolised to derivatives that are able to activate AhR. This would be in line with the supposition that the compounds that are able to activate AhR, present in the microbial supernatant originating from SHIME® fermentation, are derivatives from tryptophan.

In paragraph 3.1., three peaks were specifically named (peak A, peak B, peak C). As stated, peak A was not clearly present in the distal colon supernatant, originating from L-SHIME® fermentation. It is assumed that peak A is (the inactive form of) the enzyme pepsin. This is because pepsin was added to the stomach of the M-SHIME® system, but it has not been added to the L-SHIME® system. Pepsin, which is able to break down proteins, is naturally present in the stomach of humans and many other animals [67]. When leaving the stomach, pepsin becomes inactivated due to the higher pH. So peak A is probably not very interesting to look at in relation to the aryl hydrocarbon receptor.

More differences in the HPLC-chromatograms have been found between the descending colon supernatant, originating from M-SHIME® fermentation, and the distal colon supernatant, originating from L-SHIME® fermentation. Differences in HPLC-chromatograms in the distal colon supernatants have also been found between different donors. The gut microbiota vary between different individuals, and can fluctuate over time [68]. These variations in gut microbiota may explain the differences found between the different donors originating from L-SHIME® fermentation. The variations in gut microbiota may also explain part of the differences between L-SHIME® fermentation and M-SHIME® fermentation, since different donors have been used. However, the distinctions between the two systems probably also have a role in explaining the differences in the chromatograms. Since the gut microbiota vary between different individuals, it is hard to draw clear conclusions, because the outcome of one donor is not representative for the overall population.

The area of both peak B and peak C increased from ascending colon, to transverse colon, to descending colon. This indicates that the amount of these compounds increased when moving to a different part of the colon. The most probable reason for this is that bacteria synthesise these compounds, probably out of tryptophan, since indole is an tryptophan derivative [45]. However, it has not been proven in this study that peak B is a tryptophan derivative.

Peak B was soluble in both water fractions, indicating that peak B is a polar compound. Peak B has not been found to clearly activate AhR. However, no conclusions can be drawn out of this. While

fractionating with the HPLC, peak B was diluted with eluents. This means that it is not known whether peak B is not able to activate AhR, or whether peak B is present in a too low concentration. Peak B has also not been identified. Although peak B has not been proven to activate AhR, we still suspect that peak B is an AhR agonist. The reason for this is that peak B increases from ascending colon, to transverse colon, to descending colon, while the AhR activation also increases.

Peak C was soluble in hexane, indicating that peak C has a low polarity. Peak C was identified as indole. The hexane fraction has not been found to activate AhR, indicating that peak C, so indole, is not an AhR ligand. This was confirmed by testing the AhR activation of indole as a pure compound, it did not activate AhR. This is in contrary with literature, which states that indole is an AhR ligand [48]. In our research it was found that the AhR activation of the water-fractions was higher than the AhR activation of non-fractionated descending colon supernatant, obtained by M-SHIME® fermentation. This could indicate that indole, or other compounds that are soluble in the organic fraction, are AhR antagonists. An antagonist binds to the receptor, but does not activate it [69].

It must be noted that ethyl acetate seemed to affect the wells in which it was present, since the plastic slightly deformed. This means that the results of samples that contained ethyl acetate are not very trustworthy. This problem can be bypassed by picking another solvent with a similar polarity as ethyl acetate, but does not affect the polystyrene wells.

4.2. I3C and DIM

I3C and DIM were originally tested to find an appropriate HPLC method for tryptophan derivatives. I3C was also tested on the CALUX® assay to find out which results a weak AhR ligands gives.

I3C was not found to clearly activate AhR. But according to literature, I3C should be able to activate AhR, though it is a relatively weak ligand [70]. According to Bjeldanes et al. [71], I3C does not bind to AhR with high affinity and may not be a significant agonist of the receptor *in vivo*. I3C was not found in the supernatants obtained by SHIME® fermentation. This was expected since I3C was not present in the microbial feed. There seem to be no clues present in literature indicating that I3C can be synthesised by bacteria.

DIM was also not found to be present in any of the supernatants obtained by SHIME® fermentation. DIM is a derivative from I3C, which was also not present in the SHIME® supernatants. It was expected that DIM would not be present in the SHIME® supernatants, since I3C was not present in the microbial feed. This means that DIM could not have been formed out of I3C. DIM was also not present in the microbial feed. The AhR activation of DIM has not been tested, but according to literature DIM is a weak AhR ligand, so it would probably give a low AhR activation [72].

4.3. Tryptophan and its derivatives activating AhR

DL-Tryptophan was able to activate AhR, which is in line with the study of Heath-Pagliuso et al. (1998) [73]. This study states that L-tryptophan and D-tryptophan are both relatively weak ligands compared to β-naphthoflavone. However, D-tryptophan is more active than L-tryptophan, so D-tryptophan will have a higher AhR activation. Proteins typically incorporate only L-amino acids into their sequences [74]. Therefore, D-tryptophan is probably not present in the microbial feed. So L-tryptophan was probably one of the AhR ligands present in the microbial supernatant originating from SHIME® fermentation. However, it probably has only a minor direct effect on the activation of the receptor. This is because the AhR activation increases with a decreasing amount of L-tryptophan present, and because L-tryptophan is a weak AhR ligand.

L-Kynurenine has not been found to clearly activate AhR. According to literature, L-kynurenine should be able to activate AhR, but it is probably not a very strong ligand [75]. L-kynurenine has not been identified in the supernatants obtained by SHIME® fermentation. However, this does not mean that it was not present. Kynurenine is the first step in the kynurenine pathway, after this it is metabolised into other compounds such as kynurenic acid [41]. So L-kynurenine could already have been metabolised to other compounds. It could also have been that L-kynurenine was present in too low concentrations for the HPLC to detect. However, the chances are limited that compound(s) from the kynurenine pathway were responsible for the AhR activation of the microbial supernatant obtained by SHIME® fermentation, since L-kynurenine has not been found in the microbial supernatant.

FICZ, which is a strong ligand according to literature [31], was not found in the supernatants obtained by SHIME® fermentation. This was desired, since FICZ is formed when tryptophan comes into contact with both visible and UV-light [76]. The vessels used in the SHIME® system are transparent, so UV-light can go through the glass. When FICZ would have been formed, this would mean that the SHIME® system is not reliable, since there is no light present in the actual human gastrointestinal tract. But since FICZ is not formed, it does not seem like a problem that the vessels are transparent.

As said above, DL-tryptophan and three tryptophan derivatives (indole, L-kynurenine, and FICZ), have been tested. However, much more tryptophan derivatives activating AhR exist that are not tested. Other interesting tryptophan derivatives that are AhR ligands and can be produced by intestinal bacteria include indole-3-aldehyde, indole-3-acetic acid, tryptamine, and 3-methyl indole (skatole) [40, 45, 48]. This said, probably many compounds are yet unknown to be AhR ligands and produced by bacteria. The chemical structure of skatole is similar to indole, skatole only has a methyl-group connected to the pyrrole ring [77, 78]. This means that skatole will probably dissolve in the organic fraction (e.g. hexane and ethyl acetate), which did not have a clear AhR activation. This indicates that skatole is probably not a major AhR ligand present in the supernatant resulting from SHIME® fermentation. So it is probably not very interesting to look at skatole in this research. Indole-3-aldehyde, indole-3-acetic acid, and tryptamine have polar groups, so it is expected that these compounds will dissolve in the water fraction when mixing the descending colon supernatant originating from SHIME® fermentation with a non-polar solvent such as hexane [45]. So indole-3-aldehyde, indole-3-acetic acid, and tryptamine are interesting to look at in this research.

4.4. Microbiota that metabolise tryptophan

According to literature, the selected strains of bacteria were able to break down tryptophan. This is why these bacteria were considered interesting to research, to find out whether these bacteria can actually break down the SHIME® medium into AhR ligands.

The difference in tryptophan concentration between the SHIME® medium and the three bacteria strains in SHIME® medium is not very clear. Strain B even had a higher tryptophan concentration than the SHIME® medium itself. Therefore it can be concluded that the three bacteria strains did not seem to metabolise tryptophan. The HPLC chromatograms of the SHIME® medium and the three bacteria strains in SHIME® medium did not have big differences. Most differences were small, only one peak is clearly lower in the chromatograms of strain B and C in SHIME® medium. But since there were some differences, the bacteria did seem to have some activity, though a very low one.

The activation of the bacteria strains (confidential) in both YCFA medium and SHIME® medium was lower than the activation of the media without any bacteria. There are several situations that could have happened and be the reason of this. A first situation is that the bacteria metabolised AhR antagonists. In this way, the receptor could not have been activated. However, this does not seem

the most likely option. The activation of the media is already low, lower than 5% of the activation of β-naphthoflavone. Also, there were no big differences between the chromatograms of the media and the corresponding bacteria. A second situation is that the bacteria metabolised very strong AhR agonists present in the SHIME® medium into weaker agonist or molecules that did not activate AhR. However, this does not seem most likely either. As stated before, the activation of the media is already low, so there were probably no very strong agonists present. The ingredients of the SHIME® medium are also known, there do not seem to be any agonists present in the medium, except for the minor AhR agonist L-tryptophan. A third situation is that the bacteria seemed barely active because the concentration was too low. This actually seems to be the case. An OD600 of 0.1 is relatively low, so the bacteria probably did not grow well. This means that they produced less metabolites, so the concentration of the ligands is low. A reason for this could be that the maximum population of these bacteria was relatively low.

According to literature, bacteria species that are able to break down tryptophan into AhR ligands include *Lactobacillus reuteri*, *Lactobacillus bulgaricus*, and *Pseudomonas fluorescens* [45, 79, 80]. Tryptophanase is an enzyme that degrades tryptophan to indole, pyruvate, and ammonia [81]. As stated previously, indole is an AhR ligand according to literature [48]. However, this is in contrary with the results of this study. Still, for completeness, several (Gram-negative) bacteria species that possess tryptophanase according to the study of Martino et al. (2003) are listed: *Klebsiella oxytoca*, *Providencia stuartii*, *Citrobacter koseri*, *Morganella morganni*, and *Haemophilus influenzae* type b [82].

5. Conclusion

This study aimed to identify the compound(s) present in the microbial supernatant originating from SHIME® fermentation, that trigger the aryl hydrocarbon receptor (AhR). These compound(s) were not identified, but an indication was found that these compound(s) were, as suspected, derivatives from the amino acid tryptophan. According to literature, several tryptophan derivatives are confirmed to be ligands for AhR. Several of these tryptophan derivatives can be produced by gut microbiota.

The activation of AhR was found to increase when moving from the ascending colon and the transverse colon to the descending colon. The amount of tryptophan decreased when moving from the ascending colon to the transverse colon and the descending colon. There is possibly a causality between the found increase of AhR activation and the decrease in concentration of tryptophan. This, in turn, indicates that derivatives from tryptophan, metabolised by bacteria, activate AhR. L-Tryptophan itself seemed to have a minor direct role in the activation of AhR. The AhR ligands were found to be polar. Indole, which is a tryptophan derivative, did not seem to be responsible for the high receptor activation, but it might be an antagonist. The tryptophan derivative L-kynurenine was not found in the SHIME® microbial supernatant, but this does not have to mean that it was never present, since it can be metabolised into compounds further into the kynurenine pathway.

The tryptophan derivative FICZ, which is formed when tryptophan comes into contact with light, was also not found to be present in the SHIME® microbial supernatant. There is no light in the human gastrointestinal tract. Since FICZ was not found in the supernatant, it is not a problem that the vessels used in the SHIME® system are transparent.

6. Recommendations

A recommendation is to purchase and test several tryptophan derivatives on the HPLC. Compounds interesting to start with are indole-3-aldehyde, indole-3-acetic acid, and tryptamine. The resulting chromatograms should be compared with the chromatograms of the descending colon supernatant obtained by SHIME® fermentation, to see whether the compared pure compound is present in this supernatant. When this is the case, these compound(s) should be tested on the CALUX® assay to measure the AhR activation. This way, there can be seen whether tryptophan derivatives are actually the major ligands present in the microbial supernatant, originating from SHIME® fermentation. If tryptophan derivatives are found to activate AhR, there could also be seen which tryptophan derivatives activate AhR.

What is also recommended to test, is to make the microbial feed in such a way that no tryptophan is present, and supply this to the bacteria in the SHIME®. There are bacteria present in the gastrointestinal tract able to synthesise tryptophan [83]. Still, the bacteria will probably have much less tryptophan present to metabolise into derivatives that activate AhR. The samples resulting from this SHIME® experiment should be centrifuged and the supernatant should be filtrated. The filtrated supernatant should be tested on the CALUX® assay. If AhR has approximately the same amount of activation, there are other molecules responsible for the AhR activation.

Another recommendation is to add food products that are rich in tryptophan to the microbial feed for the SHIME® system. According to Comai et al. (2006), soybeans have a protein tryptophan content of 502mg/100g of dry flour, which is the highest of the nine tested legume seeds in that study (lupins, soybeans, groundnuts, beans, broad beans, lentils, vetches, chickpeas, and peas) [84]. Chickpeas were found to contain the highest amount of free tryptophan (58.2mg/100g dry flour). So

it would be interesting to add soybean flour and chickpea flour to the microbial feed for the SHIME® system.

The last recommendation is to test whether indole works as an AhR antagonist. Different concentrations of indole should be tested on the CALUX® assay together with 5µM β-naphthoflavone to see whether the AhR activation decreases when indole is added.

7. References

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Appendix

I. HPLC chromatograms

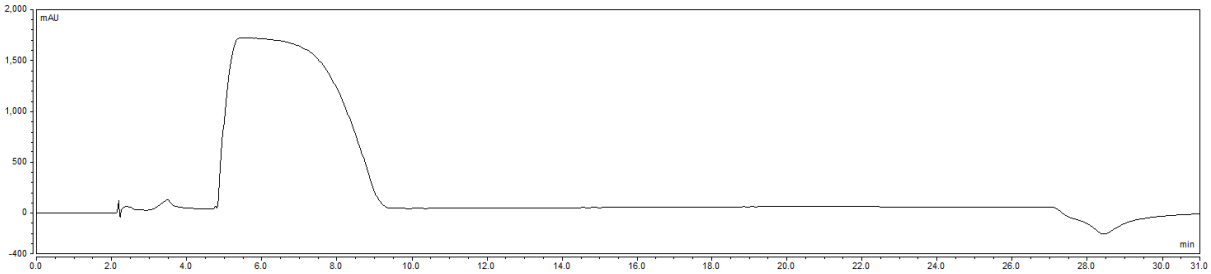

Figure 20. HPLC chromatogram of pure ethyl acetate (210nm)

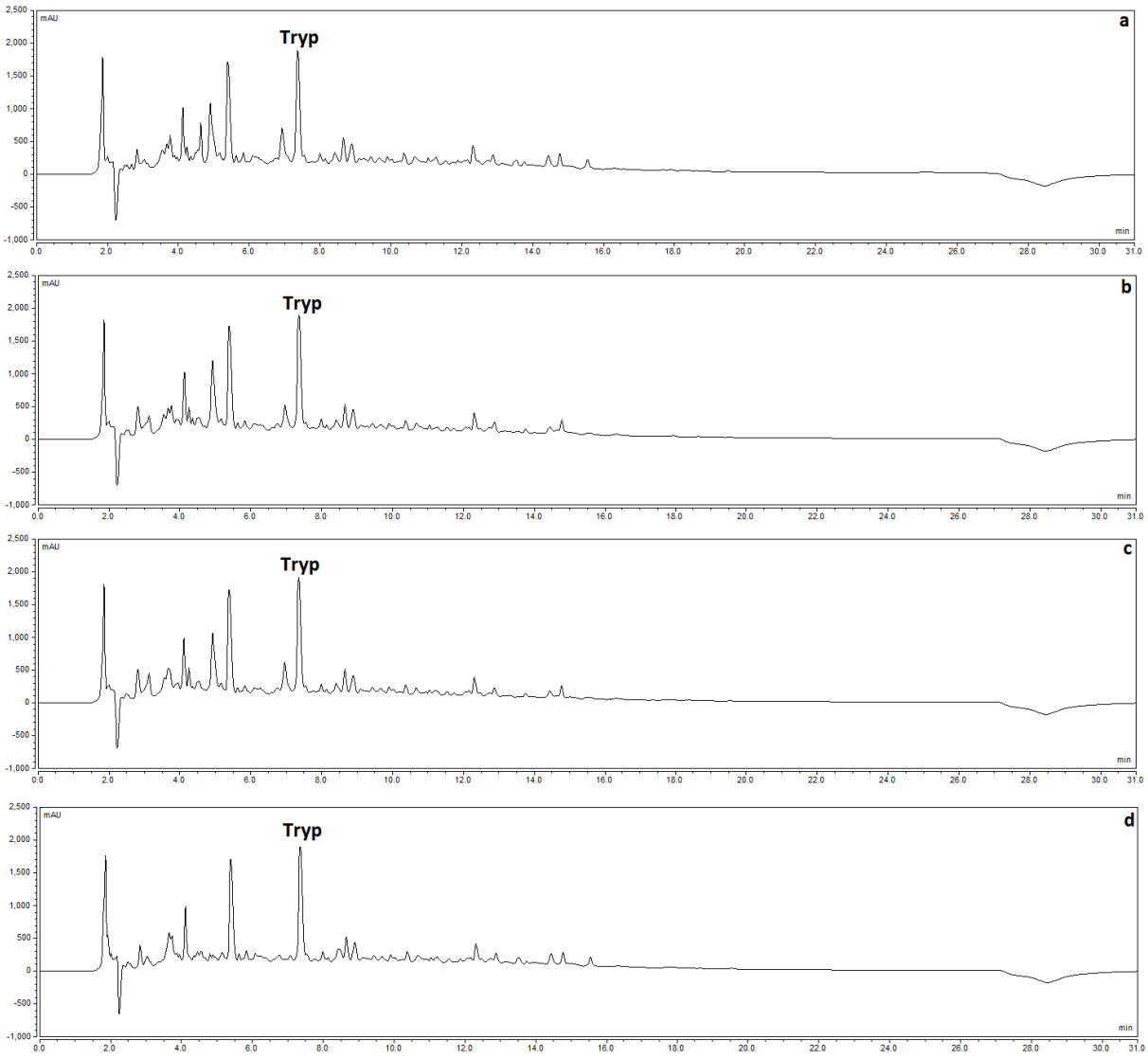

Figure 21. HPLC chromatograms (210nm)
a: YCFA medium
b: Bacteria strain 1 growing in YCFA medium
c: Bacteria strain 2 growing in YCFA medium
d: Bacteria strain 3 growing in YCFA medium
Tryp: tryptophan

II. HPLC calibration curves

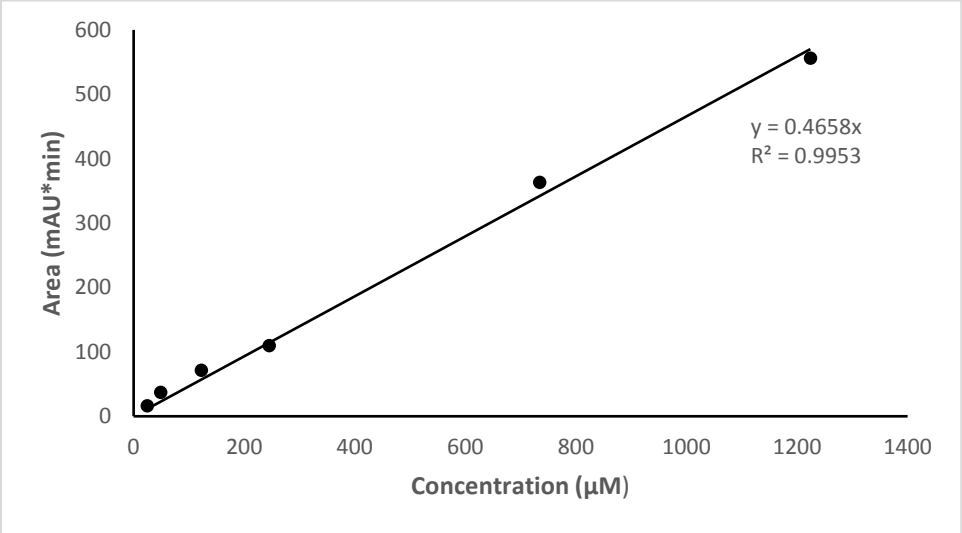

Figure 22. HPLC calibration curve of tryptophan

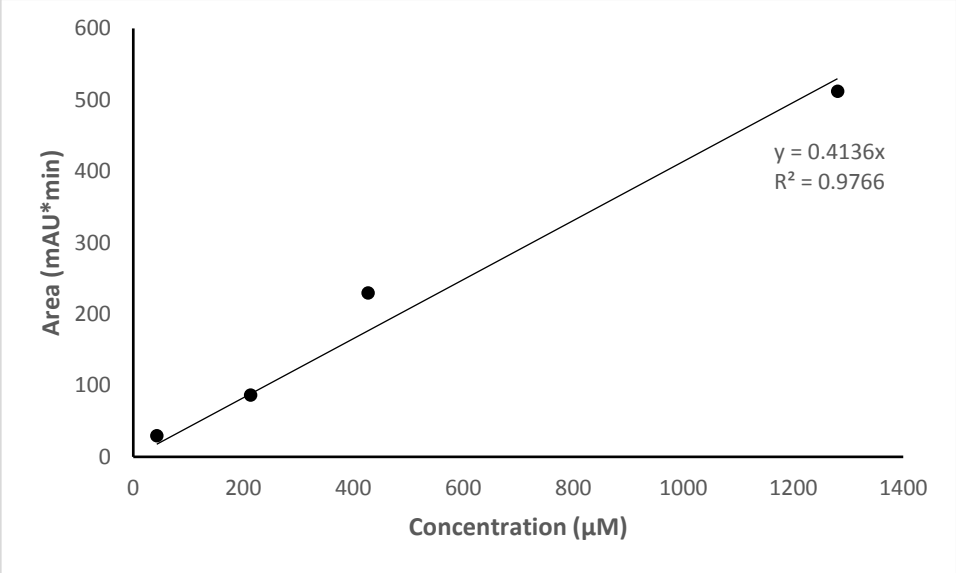

Figure 23. HPLC calibration curve of indole

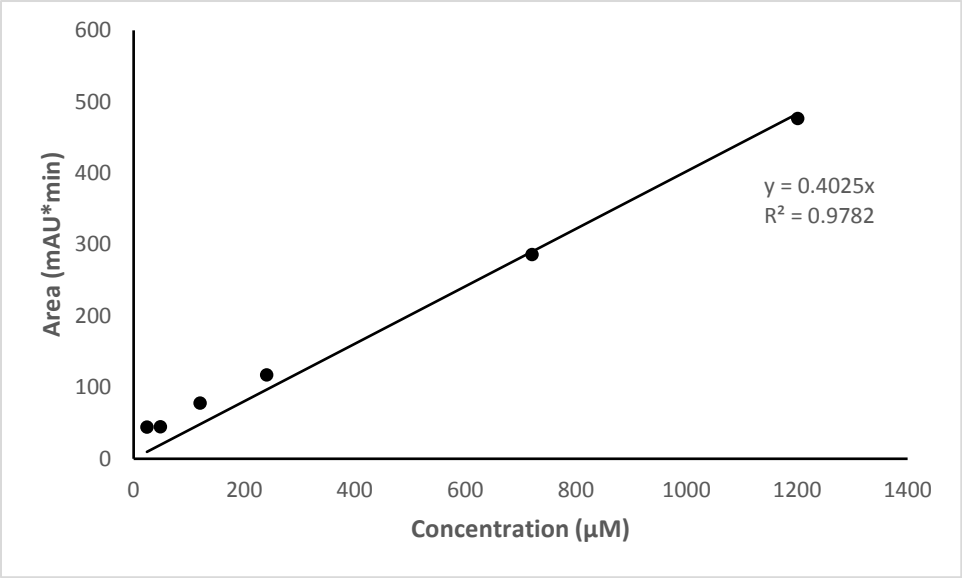

Figure 24. HPLC calibration curve of kynurenine

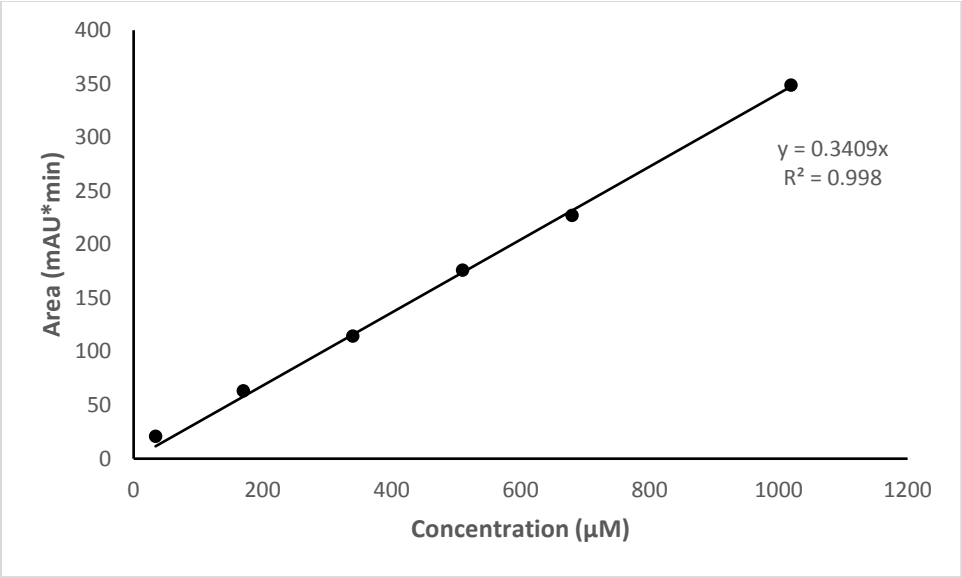

Figure 25. HPLC calibration curve of indole-3-carbinol (I3C)

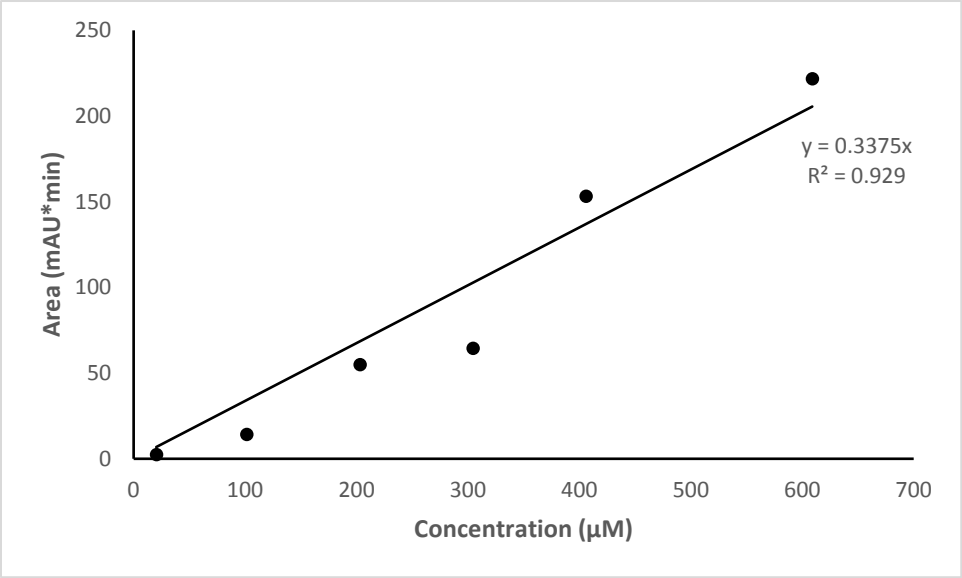

Figure 26. HPLC calibration curve of 3,3'-diindolylmethane (DIM)

III. Ethics course

IIIa. Ethische appendix: Weggegooid geld of toch een grote kans?

Tijdens mijn thesis verdiep ik mij in de "Aryl Hydrocarbon Receptor" (AhR). Dit is een receptor die aanwezig is over het hele lichaam en bij activatie gezondheidsbevorderende effecten heeft (Benson and Shepherd 2010; Berstad, Raa and Valeur 2014; Li et al. 2011; Shimada 2006; Wei et al. 2013). Enkele van deze gezondheidsbevorderende effecten zijn het onschadelijk maken van bepaalde kankerverwekkende stoffen en het remmen van de ontwikkeling van auto-immuun ziekten (zoals de ziekte van Crohn) en allergieën. Mogelijk kan activatie van de receptor ook als medicijn werken tegen auto-immuun ziekten en allergieën. Door deze gunstige effecten is er veel onderzoek naar de receptor. Enkele stoffen die de receptor activeren zijn van nature aanwezig in bepaalde voedingsmiddelen. Er wordt gekeken of deze stoffen ook aan andere voedingsmiddelen kan worden toegevoegd, waardoor dit voedingsmiddel een "functional food" wordt.

Zelf heb ik verschillende allergieën en mijn broertje heeft de ziekte van Crohn. Er zijn geen medicijnen die ervoor zorgen dat de allergie/ziekte daadwerkelijk wordt verholpen. Daarom zou het erg fijn zijn als deze stofjes zouden werken en de allergieën en ziekte zouden verhelpen.
Hoewel de gezondheidsbevorderende effecten erg gunstig lijken, is er helaas een keerzijde. Het innemen van de stofjes brengt mogelijk een verhoogd risico op kanker met zich mee (Dietrich and Kaina 2010). Met eerdere vakken heb ik geleerd dat als een stofje (mogelijk) kankerverwekkend is, het nooit toegevoegd zou mogen worden aan voedsel. Hierdoor vroeg ik mij af, zou er wel onderzoek gedaan moeten worden naar de stoffen terwijl deze waarschijnlijk nooit (extra) aan het voedsel toegevoegd mogen worden?

'Als we de AhR liganden, dit zijn de stofjes die AhR activeren, onderzoeken, zouden we kennis verschaffen over deze liganden en de receptor. Daarnaast zouden de gezondheidsbevorderende effecten worden uitgediept. Zouden we de AhR liganden niet onderzoeken, dan zou er veel (belasting)geld en moeite in een ander onderzoek kunnen worden gestoken dat vermoedelijk meer oplevert, aangezien deze stoffen waarschijnlijk nooit aan levensmiddelen worden toegevoegd door de mogelijke verhoogde kans op kanker.'

Om een zo goed mogelijke keuze te maken wordt er gebruikt gemaakt van de ethische matrix. In tabel 1 staat de matrix die is ingevuld volgens de theorie van Mepham (2013). In de tabel staan de belanghebbenden en de drie principes, die uit ethische theorieën zijn afgeleid, weergegeven. Het eerste principe, welzijn, is afgeleid uit het utilisme. Het utilisme streeft naar het grootste geluk voor het grootste aantal (Mill 1863). Of een handeling of keuze juist is, hangt dus af van de gevolgen. Autonomie is afgeleid uit de deontologie. De theorie, met als grondlegger Immanuel Kant, streeft naar een goede intentie (McNaughton and Rawling 2007). Of een handeling of keuze juist is, hangt af van het motief of de intentie. Het laatste principe is eerlijkheid/ rechtvaardigheid. Dit principe komt voor in beide theorieën, maar omvat ook de fundamentele leerstelling uit de moderne sociale contract theorie.

Zoals in tabel 1 te zien is zijn er drie betrokkenen die te maken hebben met deze kwestie. De eerste betrokken partij is de maatschappij, waarbij we naar de gemiddelde (gezonde) mens kijken. De maatschappij heeft waarschijnlijk weinig baat bij het onderzoek naar deze receptor, aangezien er waarschijnlijk niks met deze stofjes gedaan mag worden. Daarom is het voor de maatschappij eerlijker om het (belasting)geld in ander onderzoek te steken, waar de maatschappij meer baat bij heeft. Als tweede komen we bij de personen met een of meerdere allergieën en/of auto-immuun ziekten. Deze mensen hebben misschien veel baat aan het onderzoek, als mogelijk blijkt dat door het onderzoek een nieuw medicijn of "functional food" op de markt komt. De laatste betrokken partij is

de onderzoeker, die dit onderzoek uit wil voeren. Het is zijn eigen keus om dit onderzoek uit te voeren.

Tabel 2: Ethische matrix volgens Mepham (2013)

Belanghebbenden	**Welzijn**	**Autonomie**	**Rechtvaardigheid**
Maatschappij (gezonde mensen)	Het kost de maatschappij veel geld en levert weinig op	De maatschappij wil zelf kiezen waar onderzoek in gedaan wordt, en heeft hier geen directe baat bij	Het is niet eerlijk om er zoveel geld en tijd in te stoppen als het toch weinig oplevert
Mensen met een allergie/auto-immuun ziekte	Er is een kans dat het onderzoek nieuwe medicijnen (of "functional foods") oplevert, waardoor deze mensen zich beter voelen	Deze mensen willen dat er onderzoek plaatsvindt, omdat zij er misschien direct baat bij hebben	Het is eerlijk om te onderzoeken of het wellicht nieuwe medicijnen oplevert
Onderzoekers	De onderzoeker krijgt inkomen als hij het onderzoek uitvoert	De onderzoeker kiest zelf om het onderzoek te doen	Het is eerlijk om de wetenschap te laten begrijpen hoe de AhR-liganden en de receptor werken

Er is dus geen eenvoudige oplossing. Aan de ene kant staat het verlies aan kosten en tijd aan het onderzoek dat ook aan ander (belangrijker) onderzoek kan worden besteedt. Aan de andere kant is er een mogelijkheid dat de uitkomsten van het onderzoek een medicijn en/of "functional foods" opleveren. Ook is de keuzevrijheid van de onderzoeker van belang.

Volgens prof. dr. Eelke de Jong (2012) dienen wetenschappers zelf te onderzoeken wat relevant is of relevant kan zijn, ongeacht de mogelijke uitkomst. Ze dienen minder afhankelijk te zijn van de maatschappelijke druk. Prof. dr. Lex Bouter (2007) vindt dat onderzoek maatschappelijk relevant moet zijn. Ook moet de maatschappelijke relevantie van het onderzoek transparant worden gemaakt door universiteiten. Hij vindt dus dat er goed gekeken moet worden naar de relevantie van het onderzoek en dat hierover volledige transparantie naar buiten is.
Ik ben het hierin vooral eens met prof. dr. Eelke de Jong. De wetenschapper moet zelf kijken of het onderzoek maatschappelijk relevant is. Is dit niet het geval, dan zou de wetenschapper zijn onderzoek moeten veranderen zodat het wel relevant is. Wel moet de wetenschapper objectief blijven en de eerlijke uitkomst van het onderzoek naar buiten brengen, ook al blijkt deze uitkomst niet relevant te zijn.

Naar mijn mening is de AhR blijven onderzoeken het goede te doen. De uitkomst van onderzoeken kan men niet voorspellen, dus er zal altijd nieuwe kennis uitkomen. Als de kans is dat mensen van hun allergieën en auto-immuun ziekten af kunnen komen, vind ik dit de plicht van onderzoekers om dit ook daadwerkelijk te onderzoeken. Ook valt het mogelijke verhoogde risico op kanker misschien wel weg tegen de positieve effecten, bijvoorbeeld doordat het alleen schadelijk is wanneer de stofjes die de receptor activeren in te grote hoeveelheden geconsumeerd worden. Mensen kunnen dan nog

altijd zelf kiezen of ze producten met deze stoffen gebruiken. Wel zou men dan voorzichtig moeten zijn met bepaalde claims op de verpakking wanneer deze nog onvoldoende bewezen zijn. Het is natuurlijk nooit eerlijk om mensen te misleiden. Ook zou je daarmee het tegenovergestelde kunnen bereiken, dat mensen denken hun gezondheid te stimuleren, maar juist bezig zijn met de gezondheid te schaden.

Ik heb de twee kanten (wel of niet onderzoek naar AhR doen) tegen elkaar afgewogen. Ook al gaat het bij de kant met onderzoekers en mensen met een allergie/auto-immuun ziekten over minder mensen, vind ik deze redenen persoonlijk zwaarder wegen dan de redenen van de maatschappij. Dit is niet de utilistische manier om naar dit probleem te kijken, waarin er wordt gekeken naar het grootste geluk voor het grootste aantal mensen (Mill 1863). Voor het grootste aantal mensen is de beste oplossing om geen onderzoek uit te voeren naar de receptor. Toch ben ik zelf van mening dat de receptor wel moet worden onderzocht, wat de deontologische manier is om dit probleem te bekijken. De deontologische stroming vindt hier dat er een plicht is te onderzoeken of de mensen met een allergie/auto-immuun ziekte geholpen kunnen worden, waar ik het ook volledig mee eens ben (McNaughton and Rawling 2007). Verder mag je de keuzevrijheid van de onderzoeker niet negeren, wat ook een deontologische argument is. Zelf vind ik ook dat mensen met een allergie/auto-immuun ziekte er meer baat bij hebben dat de receptor wel wordt onderzocht, dan dat de maatschappij er baat bij heeft om de receptor niet te onderzoeken. Mijn conclusie is dus om onderzoeken naar AhR wel uit te voeren.

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IIIb. Wetenschapsfilosofische appendix

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Stoutjesdijk, Maartje	960618810060
Strolenberg, Willem	961104813110
Sweers, Luc	960923819050
Tjin A-Lim, Sjoera	951207836020
Veenemans, Justus	940810856050
Veldhuizen, Zoe	960804863070
Ven van der, Joris	960520866110
Verbart, Charlotte	960604868080
Vernhout, Hella	

Criteria waaraan een wetenschappelijk rapport moet voldoen

1. De wetenschapper moet objectief zijn en wanneer hij zijn mening geeft moet duidelijk worden aangegeven dat dit slechts een mening is.
2. Experimenten moeten zo opgezet worden dat getracht wordt de hypothese te weerleggen.
3. Subjectieve kennis moet worden geïntegreerd met objectieve (schriftelijke) kennis.
4. Het rapport moet een middel zijn om dichter bij de ultieme waarheid te komen.
5. Alle theorieën in het rapport moet worden beschouwd als hypothesen.
6. De wetenschappers moeten tijdens de samenwerking altijd open en eerlijk met elkaar communiceren, ook mag er niet bewust informatie worden achtergehouden. Op deze manier kunnen er geen misverstanden of miscommunicaties ontstaan.
7. De wetenschapper moet een objectieve en eerlijke uitkomst van het onderzoek boven eventuele belangen van zichzelf en de opdrachtgever kunnen zetten.
8. Alle data moet op eerlijke en de juiste manier worden verkregen.
9. Als de wetenschapper informatie gebruikt die hij niet heeft geproduceerd, moet ten alle tijden deze worden opgenomen als referentie.
10. Het rapport moet duidelijk zijn en het onderzoek reproduceerbaar.

Het dilemma, de toetsing en het interview

Ons gekozen dilemma gaat over AhR liganden, dit zijn moleculen die aan voeding of medicijnen zouden kunnen worden toegevoegd om hun functie in positieve zin te gebruiken. Als AhR liganden als medicijn of ‘functional foods’ beschikbaar worden gesteld kan dit als positief effect een verlaagd risico op auto-immuunziektes met zich meebrengen. Echter, AhR liganden geven mogelijk ook een verhoogde kans op kanker. Het is dus afwegen tussen de kans op kanker en het verlaagde risico op andere ziekten.

Na aanleiding van het interview met Karl Popper en de artikelen van de “De Groene A’dammer” en “Vrij Nederland”, hebben we criteria opgesteld waaraan een wetenschappelijk rapport zou moeten voldoen. Deze criteria hebben we getest op de thesis die de achtergrond vormt voor ons dilemma en voorgelegd aan Jonna Koper, de begeleider van deze thesis.

De belangrijkste twee criteria in overeenstemming met Jonna gaan over de manier waarop de data moet worden verkregen en de reproduceerbaarheid van het onderzoek. Alle data moet op een eerlijke manier worden verkregen en het rapport moet duidelijk en reproduceerbaar zijn. Deze twee criteria zijn deels met elkaar verbonden, de reproduceerbaarheid maakt het mogelijk om te controleren of de data op een eerlijke manier is verkregen. Zonder eerlijke data of de mogelijkheid om de data te controleren heeft het onderzoek geen waarde. De waarde in dit onderzoek wordt

gecreëerd in het schrijven van het rapport. Een exacte herhaling wordt mogelijk gemaakt door duidelijke materiaal en methode en omschrijving van de experimenten met bijbehorende resultaten. Uiteindelijk komt het neer op het vertrouwen in de onderzoeker en daarmee zijn rapport.
Het volgende criterium heeft betrekking op het berdrijfsleven. De wetenschapper moet een objectieve en eerlijke uitkomst van het onderzoek boven eventuele belangen van zichzelf en de opdrachtgever kunnen zetten. Jonna vindt dat het bedrijfsleven geen invloed mag hebben op de uitkomst, maar dit is soms moeilijk. Onderzoek word vaak voor een bedrijf gedaan en door hen gefinancierd. Duidelijke afspraken moeten worden gemaakt over het publiceren van het onderzoek, ook als dit een negatieve uitkomst geeft voor het bedrijf. Dit mogelijke knelpunt wordt hier voorkomen doordat het onderzoek niet door een bedrijf wordt gefinancierd. Een criterium dat voorkomt uit het interview met Karl Popper gaat over het gebruik van subjectieve kennis in het rapport. Subjectieve kennis mag worden gebruikt zolang het wordt geïntegreerd met objectieve (schriftelijke) kennis. Dit was het enige criterium waarmee Jonna het niet helemaal eens was. Zij denkt dat in principe alle kennis in de wetenschap objectief is en als er subjectieve kennis is, dat deze op een objectieve manier onderzocht moet worden en geïntegreerd. De betrouwbaarheid van dit onderzoek wordt juist ondersteunt door continue subjectieve kennis te integreren met objectieve kennis, persoonlijke meningen spelen geen rol. Een tweede criterium afkomstig van Karl Popper gaat over de waarheid. Het rapport moet een middel zijn om dichter bij de ultieme waarheid te komen. Ook het gebruik van AhR liganden zal een ultieme waarheid hebben, maar Jonna denkt dat deze thesis daar maar een kleine invloed op zal hebben. Er word getracht gedurende dit onderzoek om zoveel mogelijk nieuwe informatie te winnen en reeds bekende informatie wordt nogmaals bewezen. Dit zodat de ultieme waarheid dichterbij is.

De volgende vijf criteria zijn volgens ons en Jonna van gelijkwaardige toepassing als de ander criteria, maar zijn verder niet diepgaander besproken.
Ten eerste moet de wetenschapper objectief zijn en wanneer hij zijn mening geeft moet duidelijk worden aangegeven dat dit slechts een mening is. In dit onderzoek speelt de persoonlijke mening geen rol, maar hoe data geïnterpreteerd wordt natuurlijk wel. De persoonlijke invloed wordt nog beperkter door het bespreken van de interpretatie met anderen (begeleider).
Ten tweede moet de wetenschapper ten alle tijden informatie opnemen als referentie als hij deze niet zelf heeft geproduceerd. In het rapport worden alle bronnen in de referentie opgenomen op zodanige wijze dat de oorspronkelijke publicatie en/of auteur teruggevonden kan worden. Enkel de informatie die uit het eigen onderzoek voorkomt kan zonder informatie worden genoteerd.
Verder is het belangrijk dat wetenschappers tijdens de samenwerking altijd open en eerlijk met elkaar communiceren, ook mag er niet bewust informatie worden achtergehouden. Op deze manier kunnen er geen misverstanden of miscommunicaties ontstaan. Het constant uitwisselen van informatie met medestudenten en begeleiders tijdens het onderzoek voorkomt dit. De laatste twee criteria hebben betrekking op de hypothese. Alle theorieën in het rapport moet worden beschouwd als hypothesen en de experimenten moeten zo opgezet worden dat getracht wordt de hypothese te weerleggen. Tijdens dit onderzoek wordt van alle de theorieën niet aangenomen dat ze waar zijn, maar wordt juist afgevraagd óf ze waar zijn. De experimenten die worden uitgevoerd kunnen de hypothese zowel weerleggen als bewijzen. Het bewijzen van de hypothese is hierin ondergeschikt aan het achterhalen van de ultieme waarheid.
Ondanks al deze criteria miste Jonna nog een belangrijk punt, namelijk over de manier waarop het onderzoek wordt verricht. De methodes die gebruikt worden om een hypothese al dan niet te weerleggen zijn ook van belang. In humane studies en studies met dierproeven is het ook van belang of de methodes ethisch verantwoord zijn. Jonna heeft namelijk zelf ook nagedacht over de ethische aspecten in haar onderzoek. Het doel van het onderzoek is om mensen gezonder te maken zonder

medicijnen. Hiervoor heeft zij in het verleden dierproeven gedaan, maar heeft deze weten te beperken door cellen of organoïden te gebruiken. Dit is een versimpeling, maar ethisch gezien beter. Als er uiteindelijk dierproeven gedaan moeten worden zal ze dit afwegen tegen de positieve bijdrage die het onderzoek kan leveren aan de maatschappij.

Als laatste hebben we haar een vraag gesteld over de kritische houding van de wetenschapper. We wilden haar mening over de volgende uitspraak van Karl Popper: “Science may be described as the art of systematic over-simplification.” Ze is het hiermee eens en vindt hem een intelligente man. Processen in de werkelijkheid kunnen erg complex zijn, en het is onmogelijk om deze voor honderd procent uit te pluizen. Om deze reden is alles in de wetenschap een versimpeling van de werkelijkheid. Door een simpel begin waarbij bepaalde parameters weg worden gelaten, is het mogelijk om de complexiteit later te verhogen. In haar onderzoek is dit ook van toepassing. De gebruikte SHIME® methode is een versimpeling, maar zonder deze methode zou dit aspect van het onderzoek niet kunnen worden onderzocht. Vooral het beschrijven van biologische processen is lastig, ondanks de simpele methoden worden hieruit niet vaak direct harde conclusies getrokken.