UiT The Arctic University of Norway

Faculty of Health Sciences

A Pilot Study: Potential of the Probiotic Product LaBiNIC® to Inhibit Cancer Cells

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Preface

I came in contact with Veronika and the Host-Microbe Interactions and Pediatric Research group at UiT when I was considering applying for the research year in medical school, which happened in the same time frame as I was deciding on the project for my master's thesis. Veronika suggested that I do a project that could make me learn more about gut microbiota. The suggestion was intriguing as I thought the field of gut microbiota seemed engaging, and I accepted the offer.

The project description was written in the autumn of 2021, the lab work was performed in the spring of 2022, and I wrote the thesis this spring. It has been a long process, with many changes throughout the project timeline. It has been a rollercoaster with ups, downs, frustration, and a sense of accomplishment.

I express my gratitude to my main supervisor Veronika for her thorough guidance. I appreciate you always responding quickly to my questions, being specific, and teaching a medical student how academic work is done. Even though the research year did not happen, I have learned a lot that I will bring with me when I one day get into research. I am grateful for my co-supervisor Gaute Hovde Bø, who performed and provided me with the metabolomics analysis. Moreover, he helped me getting a grasp of the complex field of metabolomics.

I want to give a big thank you to Cecilie Løkke for guiding a lost amateur in the lab work. Special thanks to my boyfriend Olav for bearing my frustrations and teaching me Microsoft Excel, not having used it since secondary school.

Tromsø, May 2023

Anna Härmä

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Abstract

Background: Pediatric cancer is the prevailing cause of death among children over one year in the Western world. Chemotherapy is one of the main modalities in treating childhood cancer but it has severe side effects and long-term health consequences. Poor response to cancer therapy has been linked to disturbance in the gut homeostasis. Probiotics have been suggested to diminish chemotherapy side effects, and studies show promising results with probiotic agents inhibiting cancer cells.

Objectives: This project aimed to experimentally evaluate the anti-cancer activity of the probiotic product LaBiNIC® through two questions: 1) Would cell-free supernatant of LaBiNIC® bacterial culture decrease viability of neuroblastoma cells, a pediatric cancer cell line? 2) What potential bacterial metabolites could mediate anti-cancer effects?

Methods/materials: LaBiNIC® was incubated with neuroblastoma cell line Kelly. Viability of cell lines was measured as fluorescence readout after applying alamarBlue™ reagent that marks metabolically active cells. The study included two experiments with incubation times of 24, 48, and 72 hours and different dilutions of cell-free supernatants. Untargeted metabolomic analysis was done on whole bacterial culture of LaBiNIC® using liquid chromatography-mass spectrometry.

Results: Dilutions of LaBiNIC® cell-free supernatant (12.5% and 25%) could significantly decrease the viability of Kelly at 48 and 72 hours by 3 and 6%, respectively, and the 25% dilution by 13% at 72 hours. Neither 12.5% nor 25% suppressed the viability at 24 hours. Lactic acid was the most abundant metabolite of LaBiNIC® after 24 hours incubation. Several metabolites associated with glycolysis increased the most after 24 hours cultivation of LaBiNIC®, while amino acids decreased the most.

Conclusion: This study identified that LaBiNIC® showed a inconsistent tendency to suppress viability of Kelly cells. Lactic acid was the most abundant metabolite produced by LaBiNIC®, but it was beyond the scope of this project to characterize whether it could mediate the anti-cancer effects. Further investigations into probiotics' inhibiting effects on neuroblastoma cells should be carried out.

1 Background

Pediatric cancer is the prevailing cause of death among children over one year in the Western world. Chemotherapy, one of the main modalities in treating childhood cancer, is effective, and the survival rate is over 90%. However, cytostatic agents are toxic and result in side effects that can be traumatic for patients and their families. In addition, the long-term health consequences are often severe, with late effects such as cardiovascular, renal, and hepatic complications. Moreover, there is a heightened risk of new malignancies. New treatment strategies that can compensate for the side effects, or increase treatment responsiveness, are needed (1, 2).

A healthy gut microbiome is an integral part of a well-functioning immune system that plays a vital role in cancer prevention. Carcinogenesis, cancer progression, and poor responses to cancer therapy have been linked to systemic immunity defects and gut homeostasis disturbances. There is increasing evidence that modulating the gut microbiome may affect responses to cancer therapy and decrease side effects (3).

Lactobacilli and bifidobacteria are the most commonly used probiotic bacteria for targeted gut microbiome modulation (4, 5). Several lactobacilli and bifidobacteria strains have shown promising antitumor properties and are considered an important part of strategies for improved cancer treatment (3, 6, 7). Using metabolite profiling, this project investigated if lactobacilli and bifidobacteria-derived metabolites from specific strains potentially mediated inhibition of a pediatric cancer cell line.

1.1 Lactobacilli and bifidobacteria-based probiotics and postbiotics

Probiotics are defined by the Food and Agriculture Organization of the United Nations and the WHO as “live organisms that, when administered in adequate amounts, confer a health benefit on the host”(8).

Human symbiotic bacteria lactobacilli are a key part of probiotics (6). Lactobacilli can improve intestinal microbiota homeostasis by producing antibacterial substances, augmenting the innate immune response through activating macrophages and natural killer cells, and changing the cytokine milieu. Lactobacilli have an important role in the treatment of several diseases, among them gastroenteritis, inflammatory bowel disease, and hepatic diseases (9).

Bifidobacteria are among the keystone bacteria of the intestinal microbiota of humans and other mammals. The bacteria are especially important in newborns, whose abundance of

bifidobacteria can amount to up to 90% of the total bacterial population during lactation. Bifidobacteria have additionally shown effects in the treatment of irritable bowel disease, atopic dermatitis, and systemic lupus erythematosus, to name a few (5).

Since the invention of new methods in genomic analysis a decade ago, new bacterial strains have been discovered, making the determination of strain-specific effectiveness easier. Studies have discovered how a specific strain can be used for the treatment of a disease. In contrast, other strains won´t have the same efficacy, suggesting that probiotics are both strain- and disorder-specific (10).

Postbiotics are "metabolic products or non-viable bacterial products from micro-organisms that have biological activity in the host" (11). Postbiotics can be short-chain fatty acids (SCFAs), different biomolecules within cell-free supernatants, peptides, organic acids, etc. For example, *Lactobacillus acidophilus* strain ATCC4356 culture supernatants have been shown to inhibit human breast cancer cells (12). The use of postbiotics over probiotics can have several advantages. In a purified form, postbiotics can act more directly on specific pathways. Some other benefits of postbiotics include easier industrial production, longer shelf life, a known chemical structure, and safer drug doses. Further, probiotics that are orally ingested live microorganisms like lactobacilli and bifidobacteria could be hazardous to immunocompromised individuals such as cancer patients (13, 14).

1.2 Anti-cancer effects of lactobacilli and bifidobacteria

Both lactobacilli and bifidobacteria have documented anti-cancer effects through induction of apoptosis and inhibition of cancer cell proliferation, in addition to immunomodulation of the host response. This includes both probiotics and postbiotics-like supernatants (15).

The anti-proliferative capabilities of lactobacilli and bifidobacteria on cancer cell lines are believed to be mediated by both live bacterial cells and their cellular constituents like the cell wall and cytoplasmic fraction. Cell-free supernatants, a form of postbiotic, have been observed to be effective but require higher doses (16) (9). Due to the importance of oxidative stress in neoplastic pathophysiology, the antioxidative effects of live probiotic bacteria are also believed to play a role in the mechanism that prevents cancer cell growth (9).

One mechanism behind the direct proapoptotic effect is thought to be mediated through SCFAs and conjugated linoleic acid, metabolites produced by bacteria. SCFAs are important signaling molecules in the immune system and can affect cell death and proliferation (6). For example,

Bifidobacterium and Lactobacillus inhibit colorectal cancer growth through inhibition of inflammation and angiogenesis and protecting the intestinal barrier through SCFAs (17). Apart from this, the known cancer-acting molecules of probiotics are bigger compounds like exopolysaccharides, nucleic acids, and larger protein structures (15). These molecules are too big to be detected by metabolomics analysis which detects smaller molecules.

Based on the evidence described above, lactobacilli and bifidobacteria show potential for augmenting current anti-cancer therapies. The abovementioned mechanisms are some hypothesized ways lactobacilli and bifidobacteria could work anticarcinogenic. However, the molecular understanding of probiotics-mediated inhibition of cancer cells is incomplete and specific mechanisms of action remain unclear. (6, 9) There is a need for more studies on the effects of probiotics in cancer cell inhibition.

1.3 Profiling of small molecules by metabolomics

Metabolomics is the large-scale study of small molecules, commonly known as metabolites, which can be both substrates and products of metabolism. Production of these molecules by a cell is influenced by its genetic makeup and response to the environment. Metabolomics is often used to analyze biochemical activity of cells.

Two primary ways that are used in metabolomics are targeted and untargeted approaches. Targeted approach measures specific sets of metabolites linked to defined groups of compounds, and the same compounds from the examined biological samples are compared against these predetermined standards. The untargeted, so-called discovery approach attempts to measure as many metabolites as possible in given samples.

The combination of liquid chromatography (LC) and mass spectrometry (MS) is a popular instrumental setup used in metabolomics. Chromatography is a separation technique used to separate compounds within a complex mixture. Liquid chromatography (LC) separates molecules within a sample by making the sample interact with a mobile and a stationary phase. Analytes interact with these two phases on a chromatographic column. Compounds are separated and extracted based on their physio-chemical properties like affinity, size, or charge. High-pressure liquid chromatography (HPLC) is an advanced form of LC that uses high pressure to force samples through columns.

Mass spectrometry (MS) is an analytical technique that measures small molecules' mass-to-charge ratio. Mass spectrometers are often used with a chromatographic system. The mass

spectrometer can measure ions and their mass through several steps presented in the mass spectrum. The retention time is the compound's time spent through the mobile and stationary phases, and the fragmentation pattern is the characteristic ion pattern made by a metabolite after being subjected to fragmentation in the mass spectrometer. Using an unknown compound's retention time and its corresponding fragmentation pattern, one can identify compounds through databases and standards (18). The compounds are quantified using the number of ions with specific masses at a certain retention time. However, since there is background noise, the quantification is relative and based on relative differences between the total numbers of ions belonging to one particular mass and retention time between samples (19).

2 Project Aim

Based on current evidence, the project hypothesis was that metabolites of a probiotic product could mediate inhibition of cancer cells. This project aimed to evaluate *in vitro* the anti-cancer activity of LaBiNIC®, a commonly used probiotic composed of three stains: *B. longum subsp. infantis* Bi-26, *B. bifidum* Bb-06, *L. acidophilus* NCFM. The focus was on answering these questions:

- Would the cell-free supernatant of LaBiNIC® suppress the viability of neuroblastoma cells, a pediatric cancer cell line?
- What potential bacterial metabolites could mediate the lactobacilli-based probiotic's anti-cancer effects?

3 Materials and methods

The experiment was executed as shown in Figure 1.

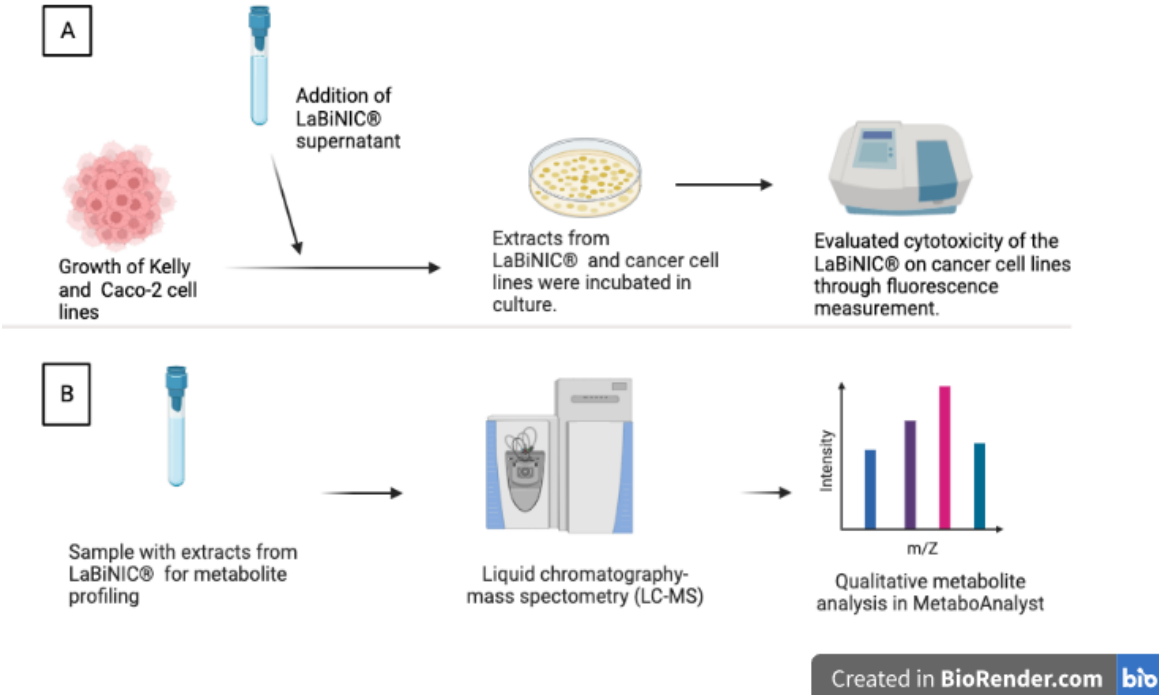

Figure* 1. *Workflow for the project. *The project was executed in two parts: A) The project evaluated whether LaBiNIC® (a probiotic including B. longum subsp. infantis Bi-26, B. bifidum Bb-06, L. acidophilus NCFM) preparations affected cancer cells proliferation- and viability B) Illustration of the metabolite profiling of the LaBiNIC® by Liquid chromatography-mass spectrometry. The data were analyzed qualitatively in the web version of MetaboAnalyst 5.0. Illustration was created in biorender.com*

3.1 Seeding out cells

The pediatric cancer cell line used was a neuroblastoma cell line called Kelly. The colorectal adenocarcinoma cell line Caco-2 was chosen as a positive control. This was because a probiotic strain *L. acidophilus* ATCC 4356, was previously documented to decrease cell proliferation and increase apoptosis (20). Neuroblastoma cell line Kelly was provided by Prof. Christer Einvik from the Pediatric Research group at the UiT Arctic University of Norway, while the Caco-2 line was provided by the research group for Host-Microbe Interaction (UiT).

The cells were grown in 1640 Roswell Park Memorial Institute (RPMI) Medium supplemented with 10% sterile-filtered fetal bovine serum (FBS) on 24-well plates. About 90 000 cells were seeded in each Kelly well. For Caco-2, there were 50 000 cells per well.

The total volume of the cells and medium in each well was 500 uL. The 24-well plates with the cells were incubated for 24 hours at a temperature of 37°C and CO_2 5% in a humified incubator (Thermo Fisher Scientific Heracel vios 250i CO_2 Incubator, Waltham, USA).

3.2 Preparation of overnight cell cultures

Kelly and Caco-2 were cultured with an extract of LaBiNIC® cell-free supernatant (21, 22). As a negative bacterial control, *E. coli* strain ATCC25922 was used. This strain has no documented anti-tumor properties. The bacterial strains were provided from the strain collection of Host-Microbe Interactions research group. The cell-free supernatant of LaBiNIC® and *E. coli* were prepared by Gaute H. Bø by using stationary phase overnight cultures, centrifugation, and sterile filtering of the resulting supernatant via 0.2 µm filter.

RPMI-1640 media (Thermo Fisher) was used to make five dilutions of LaBiNIC® bacterial supernatant: 3.125%, 6.25%, 12.5%, 25%, and 50%.

The *E. coli* supernatant was used in one dilution, which was 50%. Each experiment had three replicates of every dilution combined with Kelly and Caco-2, and RPMI media was included as an additional control. 24-well plates with LaBiNIC® together with Kelly and Caco-2, respectively, were incubated for 24 hours at a temperature of 37°C and 5% CO2 in a humified incubator (Thermo Fisher Scientific Heracel vios 250i CO_2 Incubator, Waltham, USA).

3.3 Determination of cancer cell lines viability

To investigate the viability of the cancer cells after being incubated with LaBiNIC®, fluorescence reading was used. After 24 hours incubation, each well was added 50 uL alamarBlue ™ Cell Viability agent (Thermo Fisher). AlamarBlue ™ is reduced by metabolic activity in living cells, leading to a color change from blue to pink. The intensity of the color change is proportional to live cells present and can be used in a fluorescence assay. The more live cells present, the higher the fluorescence number will be.

The 24-well plates with selected cell lines and added alamarBlue ™ were incubated for an additional three hours at 37°C and CO2 of 5% in a humified incubator. This was according to the alamarBlue™ original protocol (23). The fluorescence was measured after pipetting 100 uL from each well into a blacked-walled 96-well reading plate. Fluorescence measurements were done at 540 nm excitation and 590 nm emission wavelengths in a CLARIOstar® Plus microplate reader. The fluorescence reading was to evaluate the cancer cell lines' viability under different experimental conditions.

3.4 Metabolite profiling

The metabolomics analysis was performed by Gaute Hovde Bø. Shortly, overnight culture of 15 ml was first incubated anaerobically in gut microbiota media (24) at 37°C for 16 hours. An aliquot of the overnight culture (5 ml) was used to inoculate 100 mL of fresh media and was cultivated for 24h. The LaBiNIC® bacterial culture was sampled two times, at time point 0 (time of inoculation) and at 24 hours. The samples were characterized by LC-MS-based untargeted metabolomic analysis according to previously described protocol (25).

The metabolite analysis was carried out using the following databases: ChemSpider (26), mzCloud(27), and mzVault (in-house database). The data was filtered, and metabolites were identified using the Schymanski method (28). Dry weight of the samples was measured after drying corresponding aliquots of the samples and subtracting the dry weight of the filters. The raw metabolomics data were then normalized by using dry weight of each sample as a normalization coefficient. The metabolomics data of T0 and T24 were compared against each other to evaluate the relative difference in abundance of the compounds.

To find which metabolic pathways were active in LaBiNIC®, metabolic pathway analysis was done using the web-based tool MetaboAnalyst 5.0 (29). This was to find possible metabolic pathways from LaBiNIC® that could be linked to inhibiting the cancer cells.

3.5 Statistical analysis

Data analysis on fluorescence measurements of the cancer cell lines was carried out using the data analysis package in Excel. The different dilutions were compared to the RPMI group using student´s t-test.

Doing metabolomics can be uncertain; the second replica of the LaBiNIC® metabolomics analysis at the 24-hour timepoint fell out. For this reason, the metabolomics data were analyzed mostly qualitatively without any statistical or thorough quantitative analysis.

4 Results

4.1 Neuroblastoma cell line inhibition by LaBiNIC®

This work aimed to examine if cell-free supernatant of LaBiNIC® overnight culture can suppress the viability of neuroblastoma cell line Kelly. Two tests were possible to do within the project timeline. The first test was done with five dilutions of the LaBiNIC® culture supernatant. RPMI media was used to measure background fluorescence, and *E. coli* ATCC 25922 was used as a control to compare with the effects of common commensal gut bacteria. The initial test showed that the viability of the neuroblastoma cell line was reduced marginally with the LaBiNIC® cell-free supernatant dilutions 3%, 6.25%, 12.5%, and 25%, but not with 50% (**Figure 2A**). The 50% dilution, on the other hand, showed increased fluorescence and seemingly promoted viability.

Although Caco-2 cell line was previously documented to be inhibited by a probiotic strain of *L. acidophilus* (20), the LaBiNIC® supernatant did not seem to have such effect (**Figure 2B**). *E. coli* ATCC 25922 enhanced the viability of both the Kelly and the Caco-2 cell line by 19% and 38%, respectively.

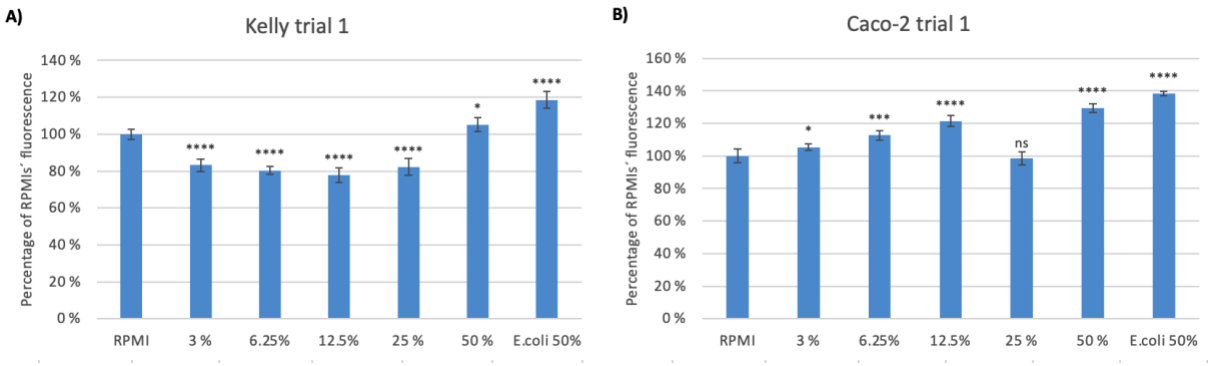

Figure 2. Initial test of bacterial inhibition of the cancer cell lines. *Mean and standard deviation of the fluorescence of the neuroblastoma cell line Kelly (A) and the colorectal adenocarcinoma cell line Caco-2 (B) after 24 hours incubation, as determined by fluorescence which viability assay. The x-axis shows the different dilutions of LaBiNIC® culture supernatant and E.coli ATCC 25922 50% culture supernatant. The y-axis represents the percentage of RPMI, with the RPMI control set at 100%. P-values were determined using student´s t-test when comparing one group to the RPMI group at the same time point. One asterisk: $p < 0.05$, two: $p < 0.01$, three: p: 0.001, and four: 0.0001. Not significant numbers with p-values > 0.05 are marked with ns.*

The positive control RNA polymerase I inhibitor CX-5461 was included in the second experiment. This compound has been shown to inhibit neuroblastoma cell growth (30). In addition, the 3% dilution was removed. This was to have room for CX-5461, while the distinction between the 3% and 6.25% dilution was minimal. Furthermore, three consecutive fluorescence measurements were executed to see the effect of the incubation time. There was one fluorescence measurement at 24 hours, one at 48, and one at 72 hours.

As **Figure 3A** illustrates, at 72 hours, the 25% LaBiNIC® supernatant significantly decreased Kelly's cells' viability by 13%. The 50% LaBiNIC® decreased Kelly´s viability by 6%, though insignificantly. The other dilutions only affected the viability of Kelly marginally, as compared to the media control. The CX-5461 progressively decreased Kelly´s cell viability by 16% at 24h, 50% at 48h, and 77% at 72h.

The effect of all measured dilutions of LaBiNIC® supernatant on the Caco-2 cell line was to increase cell viability. As shown in **Figure 3B**, this followed a dose-dependent trend of increasing viability with increasing supernatant concentration. This trend was diminished at 48h and 72h. CX-5461 significantly decreased the Caco-2´s viability by 21% at 24h, 7% at 48h, and 50% at 72h.

In the second trial, *E. coli* ATCC25922 supernatant was contaminated by bacterial cells, although it was meant to be sterile filtered. Consequently, *E. coli* supernatant was removed from the experiment.

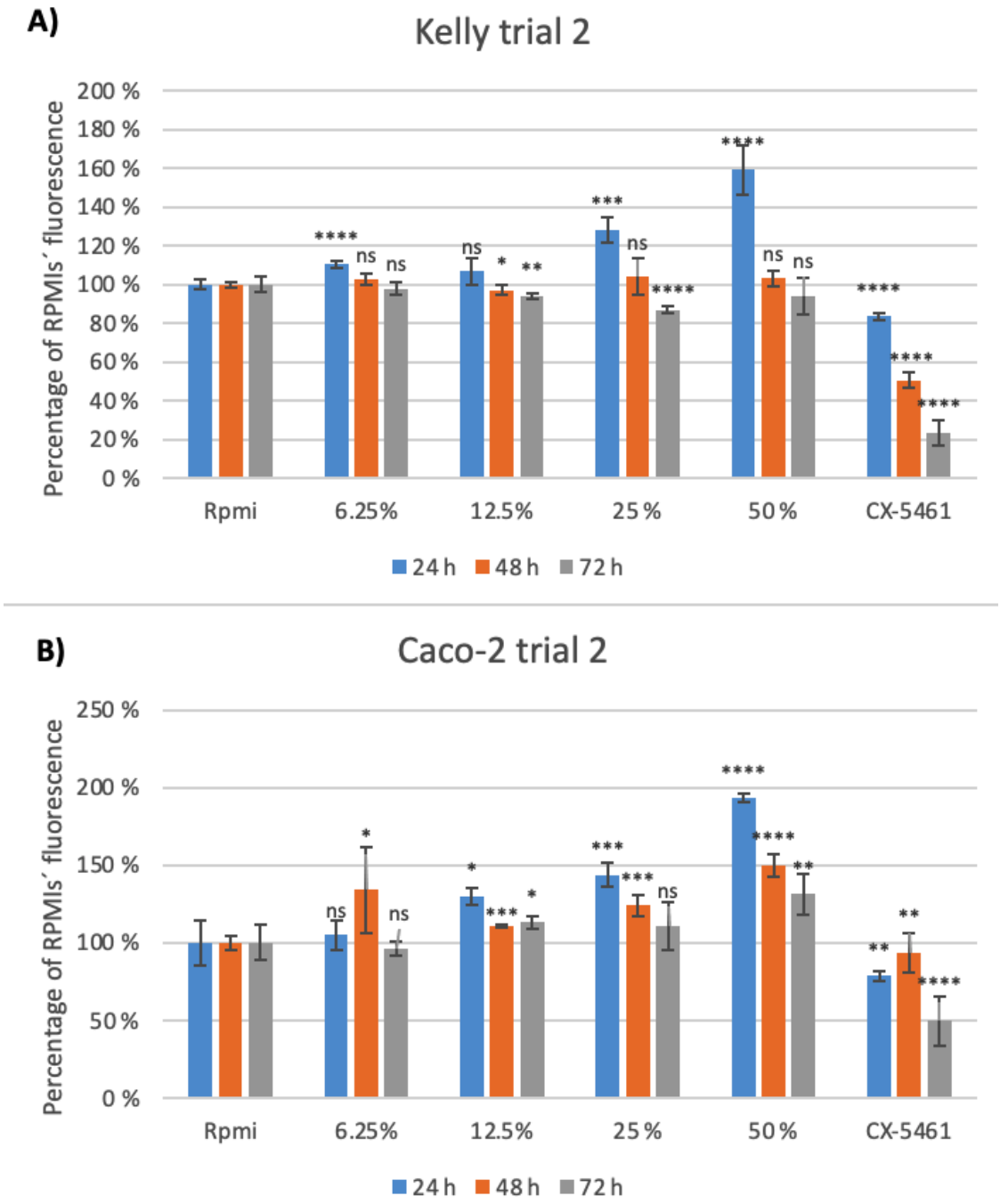

Figure 3. Viability of the cancer cell lines when incubated with different bacterial cell extracts. *Mean and standard deviation of the fluorescence of the neuroblastoma cell line Kelly (A) and the colorectal adenocarcinoma cell line Caco-2 (B) after 24, 48, and 72 hours. The X-axis shows the RPMI, different dilutions of LaBiNIC® as well as the RNA Polymerase I inhibitor CX-5461. The y-axis represents the percentage of RPMI background fluorescence, with the RPMI control set at 100%. The significance is illustrated with asterisks. The p-values were found by doing a student's t-test comparing one group to the RPMI group at the same time point. One asterisk: $p < 0.05$, two: $p < 0.01$, three: p: 0.001, and four: 0.0001. Not significant numbers with p-values > 0.05 are marked with ns.*

4.2 Metabolite profiling of LaBiNIC® bacterial culture

The metabolomics of LaBiNIC® bacterial culture was performed by the co-supervisor Gaute H. Bø using untargeted LC-MS workflow described earlier (25). Timepoints 0 hours (T0 - inoculation) and 24 hours (T24 - stationary phase culture) were selected for the analysis to evaluate metabolite production.

The metabolites were identified using three databases (ChemSpider, mzCloud, and an in-house database), filtered by confidence value of their identification, and metabolites originating from the culture media were excluded. The final list included 185 metabolites identified with good confidence. To narrow down the metabolite analysis, the focus was on the matches identified with the highest confidence, resulting in 46 metabolites. These metabolites were sorted into chemical taxonomy classes and matched with their common name (**Table 1**). All metabolites were assigned relative quantitative values based on their total ion count (TIC). The values were normalized for biomass at the different sampling points using the dry weight measurement of the bacterial culture.

Four out of ten most abundant compounds of LaBiNIC® at T24 were within the amino acids and derivates class: glutamate, aspartate, phenylalanine, and norleucine (**Figure 4**). However, all these compounds decreased from T0 to T24, indicating consumption of these amino acids by the LaBiNIC® strains.

Next, the relative decrease and increase of compounds during LaBiNIC® cultivation was evaluated based on the comparison of the peak intensity values between T0 and T24 for individual compounds. Four of the ten compounds that decreased the most from T0 compared to T24 were within the amino acids and derivates class (**Figure 5**). These were n-acetyl alanine (decreased by 93% compared to T0), n-acetyl aspartate (93%), n-acetyl glutamate (87%), and n-acetyl glycine (84%). The most decreasing compounds were n-acetyl alanine and n-acetyl aspartate, with a 93% decrease each.

Three out of ten compounds that increased the most from T0 to T24 were part of sugars and derivatives class; these were fructose-6-phosphate (increased by 441% compared to T0), 3-phosphoglycerate (278%), and 2-phosphoglycerate (70%). These are all intermediates in glycolysis. Uridine monophosphate (1318%) and cytidine monophosphate (826%) increased the most from T0 to T24, indicating their production by the LaBiNIC® strains (**Figure 6**).

Table 1: LaBiNIC® metabolites detected with high confidence and their chemical taxonomical classes according to the Chemical Entities of Biological Interest web database.

Compound	Common name	Chemical taxonomy class
HYDROPHENYLLACTIC ACID	Hydroxyphenyllactic acid	Other carboxylic acids and derivatives
DL-3-AMINOISOBUTYRIC ACID	3-Aminoisobutanoic acid	
PHOSPHOENOLPYRUVATE	Phosphoenolpyruvic acid	
CIS-ACONITIC ACID	cis-Aconitic acid	
3-PHENYLLACTIC ACID	3-Phenyllactic acid	
L-(+)-LACTIC ACID	L-Lactic acid	
PROLINE	4-Oxoproline	Proline and derivatives
ASPARTATE	L-Aspartic acid	Amino acids and derivates
N-ACETYLGLYCINE	Acetylglycine	
GLUTAMATE	L-Glutamic acid	
PHENYLALANINE	L-Phenylalanine	
N-ACETYLASPARTATE	N-Acetyl-L-aspartic acid	
NORLEUCINE	L-Norleucine	
N-ACETYLSERINE	N-Acetylserine	
N-ACETYLGLUTAMATE	N-Acetyl-L-glutamic acid	
BETA-ALANINE	beta-alanine	
N-ACETYLALANINE	N-Acetyl-L-alanine	
D-(-)-GLUTAMINE	D-glutamine	
TRYPTOPHAN	L-Tryptophan	
ADENINE	Adenine	Purine nucleotides
ADENOSINE-MONOPHOSPHATE	Adenosine monophosphate	
ADENOSINE TRIPHOSPHATE	Adenosine triphosphate	
BETA-GLYCEROPHOSPHATE	beta-Glycerophosphoric acid	Glycerophospholipids
URIDINE MONOPHOSPHATE	Uridine 5'-monophosphate	Pyrimidine nucleotides
URIDINE DIPHOSPHATE GLUCOSE	Uridine diphosphate glucose	
URIDINE DIPHOSPHATE-N-ACETYLGLUCOSAMINE	Uridine diphosphate-N-acetylglucosamine	
CYTIDINE MONOPHOSPHATE	Cytidine monophosphate	
OXOGLUTARATE	Oxoglutaric acid	Keto acids and derivatives
PYRUVIC ACID	Pyruvic acid	
SORBOSE	L-Sorbose	Sugars and derivatives
FRUCTOSE 6-PHOSPHATE	Fructose 6-phosphate	
ALPHA-D-GLUCOSE-1,6-BISPHOSPHATE	alpha-D-Glucose 1,6-bisphosphate	
3-PHOSPHOGLYCERATE	3-Phosphoglyceric acid	
2-PHOSPHOGLYCERATE	2-Phosphoglyceric acid	
METHYL GALACTOSIDE	Methyl beta-D-galactoside	
DODECYL SULFATE	Sodium Dodecyl Sulfate	Unknown
NP-016455	NP-016455	
2-HYDROXYVALERIC ACID	2-Hydroxyvaleric acid	Fatty Acyls
2-HYDROXYCAPROIC ACID	2-Hydroxycaproic acid	
CITRACONIC ACID	Citraconic acid	
16-HYDROXYHEXADECANOIC ACID	16-Hydroxyhexadecanoic acid	
PALMITOLEATE	Cis-9-palmitoleic acid	
ELAIDIC ACID	Elaidic acid	
3-HYDROXY-2-METHYLPYRIDINE	3-Hydroxy-2-methylpyridine-4,5-dicarboxylate/ 2-Methyl-3-hydroxy-5-formylpyridine-4- carboxylate	Pyridines and derivates
BENZOATE	Benzoate	Benzene and substituted derivatives
ISOPHTHALIC ACID	Isophthalamide	

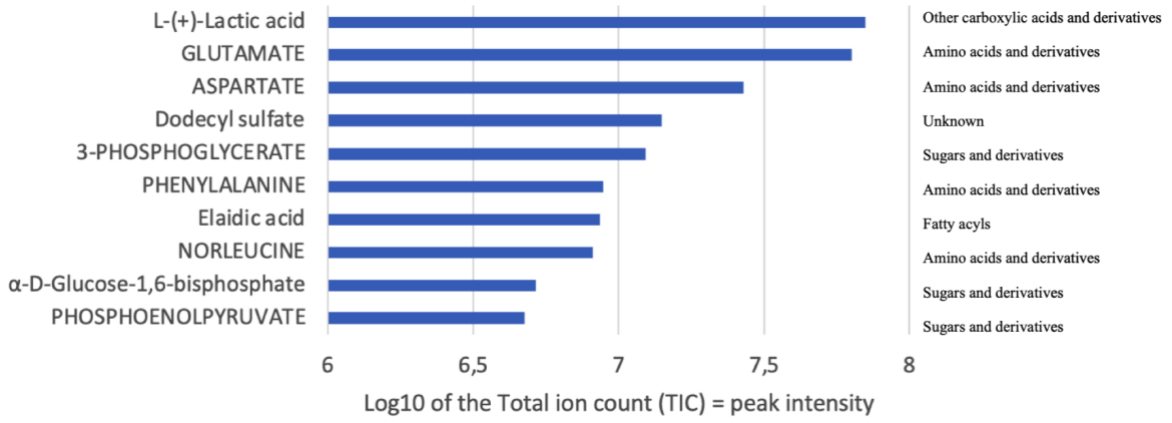

Figure 4. Ten most abundant compounds identified in LaBiNIC® culture grown for 24 hours*. The peak intensity of each compound has been log10 transformed. The peak intensity is based on the total ion count (TIC); TIC is the number of ions with specific masses at a certain retention time. To the right, on the same level, is the taxonomical class the metabolite belongs to.*

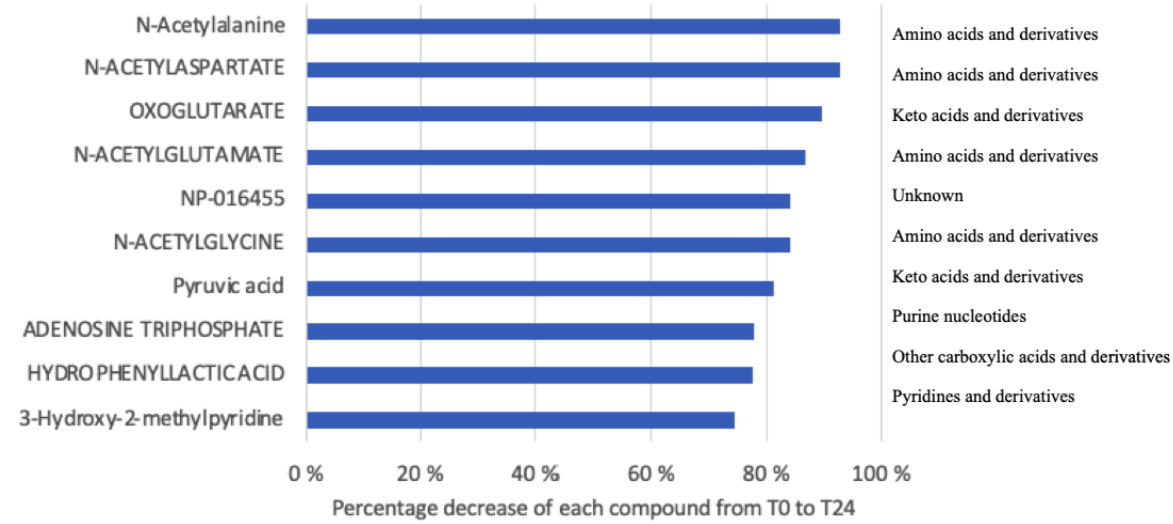

Figure 5. Ten most decreased metabolites in LaBiNIC® at time point 0h, as compared to 24h. *The x-axis shows the decrease of compounds in percentage of its T0 peak intensity, 0% means there has been no change in the compound from T0 to T24. The peak intensity is based on the total ion count (TIC); TIC is the number of ions with specific masses at a certain retention time. To the right, on the same level, is the taxonomical class the metabolite belongs to.*

To evaluate which metabolic pathways are active in LaBiNIC® during anaerobic cultivation in media mimicking the gut chemical environment (24), pathway analysis was performed by MetaboAnalyst. The data input into MetaboAnalyst´s pathway analysis was a list of all 46 compounds identified with high confidence by LC-MS analysis.

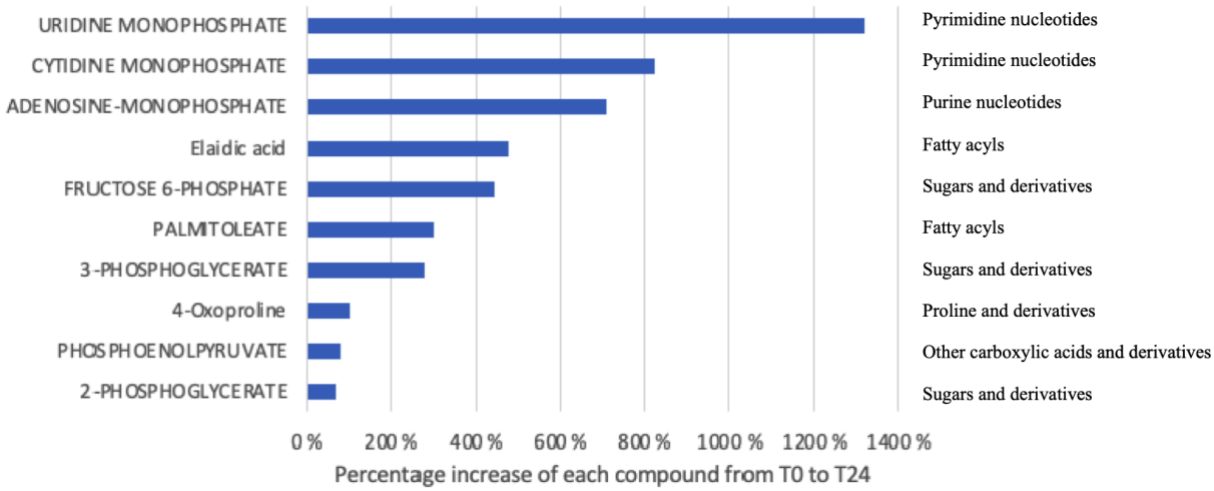

Figure 6. Ten metabolites were detected in LaBiNIC® culture that increased the most during 24h cultivation. *The x-axis shows the increase of compounds in percentage of its T0 peak intensity, 0% means there has been no change in the compound from T0 to T24. The peak intensity is based on the total ion count (TIC); TIC is the number of ions with specific masses at a certain retention time. To the right, on the same level, is the taxonomical class the metabolite belongs to.*

Because there was no option in MetaboAnalyst to choose lactobacilli or bifidobacteria (LaBiNIC® species) in the pathway library as a reference metabolome, *Staphylococcus aureus* was selected as a reference being a gram-positive bacterium as bifidobacteria and lactobacilli. Unnamed structures from our data not found in the pathway library were excluded.

As illustrated in Table 2, three significant pathways were found: alanine, aspartate, glutamate metabolism, arginine biosynthesis, and citrate cycle (TCA cycle). MetaboAnalyst provides a pathway impact score by assessing the relative importance of metabolites based on their contributions to a given biological pathway or process based on statistical analysis (31). The pathways with the highest impact score, with an adjusted p-value <0.05, were interpreted as the most important ones.

Table 2. Results from pathway analysis in MetaboAnalyst of metabolites from LaBiNIC®. *The three significant pathways with a p-value of <0.05 were assessed by the Holm-Bonferroni method (Holms adjusted p). Total is the total number of compounds in the pathway. Hits are the matched compounds from the data uploaded. Raw p is the raw p-value. Impact score is the pathway impact score calculated by MetaboAnalyst as described above.*

	Total	Hits	Raw p	Holms adjusted p	Impact score
Alanine, aspartate and glutamate metabolism	21	6	9.68E-06	7.39E-04	0.94
Arginine biosynthesis	16	5	3.88E-05	2.87E-03	0.19
Citrate cycle (TCA cycle)	20	5	1.28E-04	9.33E-03	0.20

5 Discussion

This pilot study aimed to evaluate if cell-free supernatant of probiotic product LaBiNIC® could decrease viability of neuroblastoma cells and investigate the metabolome of a stationary phase culture of LaBiNIC®.

5.1 Potential inhibiting properties of LaBiNIC® toward cancer cells

This study found contradictory results regarding the inhibitory effect of LaBiNIC® on neuroblastoma cell line Kelly viability. The initial test found that 3%, 6.25%, 12.5%, and 25% dilution of LaBiNIC® could significantly suppress Kelly cells' viability after 24 hours incubation by 17%, 20%, 22%, and 18%, respectively. In the second trial, 12.5% of LaBiNIC® cell-free supernatant significantly suppressed the viability of the neuroblastoma cell line Kelly by 3% at 48 hours and 6% at 72 hours but not at 24 hours. The 25% dilution suppressed the viability of Kelly with 13% at 72 hours but showed no suppression at 48 or 24 hours,

The results of the first and the second trial of LaBiNIC® supernatant against neuroblastoma cell line Kelly were slightly different, which might have several explanations, mostly concerning the experimental setup. However, the results of LaBiNIC® effect on Caco-2 cells were more similar in the two trials (**Figure 2, Figure 3B**), indicating good reproducibility of these findings. In general, experiments like this should be carried out several times to standardize conditions. In the future, one could count bacterial colonies or measure bacterial concentration with a spectrophotometer to standardize the bacterial culture used for producing cell-free supernatants.

The lack of inhibition at 24 hours in the second trial might suggest a need for longer incubation times in future experiments. However, Kelly was inhibited after 24 hours in the first trial. It was not within this project's scope to evaluate differences in using bacterial cultures from different growth phases. It is possible that the results would be different when using supernatant from exponentially growing bacterial cells. This assumption is based on the fact that the metabolome of actively growing cells differs from those in the stationary phase (32).

A peculiar result was that at 24 hours in both trials, the 50% dilution increased Kelly´s and Caco-2´s viability instead of decreasing it. One reason might be that this dilution indeed increased the viability of the cancer cells. Another reason might be increased toxicity of the probiotic, which can augment metabolic activity in the cancer cell. Alamarblue is, as mentioned in the method part, reduced by metabolomic activity. If LaBiNIC® is toxic, the cancer cell

might increase its metabolism to fight the toxic compound (33). The latter hypothesis is supported by the observation that this effect was reduced with increasing incubation times

E.coli ATCC 25922 enhanced both cancer cell lines' viability in the first experiment. *E.coli* supernatant was not sterile in experiment number two, leading to exclusion of these results. Also, only 50% dilution was used for *E.coli*. Further testing is needed to confirm and extend these observations, whether *E.coli* ATCC 25922 cell-free supernatant indeed enhances viability of the tested cancer cell lines.

Chen et al. found that *L. acidophilus* NCFM, part of the LaBiNIC® product, suppressed murine colon adenocarcinoma CT-26 tumor growth and induced tumor tissue apoptosis (34). In contrast to the presented in vitro study of LaBiNIC®, the study inoculated mice with live cells of *L. acidophilus* NCFM. Using dead bacterial supernatants, as in this pilot study, often requires higher dosages (9). The study by Chen et al. is one of few studies done on *L. acidophilus* NCFM and cancer cells. Though the authors found that *L. acidophilus* NCFM could induce tumor tissue apoptosis, their study design differs by using live bacteria, *in vivo* conditions, and murine colon cancer. Like the study above, Legesse Bedada et al. showed in a review from 2020 that most studies on cancer-inhibiting properties of probiotics are done with live bacteria, and the focus is mainly on colorectal cancer (15).

In our experiment, the colon cancer cell line Caco-2 did not show decreasing viability with increasing concentrations of LaBiNIC®. One study by *Dallal* et al. found that cell-free supernatant of *L. acidophilus* ATCC 4356 could reduce proliferation and induce apoptosis of Caco-2 (20). The strain used was a single probiotic strain and a different strain than in the LaBiNIC® probiotic mix. Moreover, different assays were used to measure viability, further hindering direct comparison between the presented study and the one described.

A meta-analysis by McFarland et al. from 2018 stated that the use of probiotics is both strain and disease-specific (10). That implies that it might be another probiotic strain that acts more inhibitory on neuroblastoma cells than the ones used in LaBiNIC®. In addition, LaBiNIC® is a mix of three different strains, which might have impacted each other's growth or mechanism of action. That strains in a multi-strain probiotic can affect each other in this way has been found previously (35). A recent review shows that studies done using a mix of bacterial strains are a problem, making it challenging to prove the effects of single strains (5).

Finally, this pilot was an *in vitro* study. In living organisms, the microbiota of the gut is a complicating factor. Even though LaBiNIC® appeared to have a significant inhibiting effect on neuroblastoma cells, the results are not directly comparable to *in vivo* experimental settings.

5.2 Metabolomics and pathways of LaBiNIC®

That lactic acid was the most abundant compound in LaBiNIC® was not surprising. Both lactobacilli and bifidobacteria produce lactic acid (36). One surprising result was that lactic acid decreased by 50% from 0 to 24 hours. The acrylate pathway uses lactic acid as a substrate for SCFA production, and it is a known pathway in human gut bacteria (37). It might have happened in our metabolomics analysis that the bifidobacteria used the lactic acid as a substrate; thereby, the lactic acid levels fell.

Lactic acid has been implied as a possible target therapy for inflammation-related diseases like cancer in a recent review (38). Local release of lactic acid by the cancer cells in the tumor microenvironment is associated with cancer progression, as shown in a review by Zhou et al. in 2022 (39).

Potentially, the lactic acid production by LaBiNIC® probiotic mix could influence the cancer cells externally and contribute to their increased viability. On the other hand, lactic acid-producing bacteria have been shown to inhibit cancer cell proliferation (15). Furthermore, a review from 2023 by Byun showed that studies that have examined cancer-progressing lactic acid have only investigated tumor-derived lactic acid (38). External lactic acid might not influence the cancer cells in the same way.

Alanine, aspartate, and glutamate metabolism appeared to be significantly enriched within the metabolism of LaBiNIC® bacterial culture. In line with this, most decreased metabolites after 24h of cultivation were amino acids, indicating their consumption by LaBiNIC® strains. Lin et al. found in a 2022 study that increased aspartate levels were a risk factor for prostate and breast cancer, though as a long-term risk factor. This was not the case with glutamate, which was also examined (40). The connection between aspartate levels and neuroblastoma cells is yet to be discovered.

The arginine biosynthesis pathway was one of the significantly enriched pathways with a high impact score (Table 2). Arginine is not an essential amino acid for cells, but semi-essential to cancer cells in a way that cancer cells often seem “addicted” to arginine. Arginine depletion therapy has already been established as a treatment for acute lymphoblastic leukemia. A 2021

review by Chen et al. found that arginine depletion induces apoptosis in many cancer types, including accelerating glioblastoma cell death when combined with a cytostatic agent (41). Glioblastoma is like neuroblastoma, a neural cancer, and this might suggest that arginine depletion can act as apoptosis-inducing on neuroblastoma cells. The metabolite analysis in this study found other metabolites within the arginine biosynthesis pathway but not arginine itself. It might be that the arginine biosynthesis pathway was not active in LaBiNIC® or that the untargeted metabolomics did not identify it. Moreover, the role of arginine depletion in neuroblastoma cells is not known.

One factor that made the pathway analysis less accurate is that *Staphylococcus aureus* N315 was used as a pathway library. Even though it is a gram-positive bacterium like lactobacilli and bifidobacteria, it likely has differences in metabolic pathways. For future experiments, it would be helpful to have a reference database for the strains used in LaBiNIC®.

This study did untargeted metabolomics. Of the few studies in this niche field, the most mentioned metabolomics methods targeted SCFAs. Several studies have shown that SCFAs produced by bifidobacteria and lactobacilli act inhibitory on cancer cells (17, 42). However, measurement of SCFAs requires targeted metabolomics method (18). It would be interesting to test the effect of SCFAs alone on cancer cell viability and which SCFAs are produced by LaBiNIC® strains, individually and as combined culture.

5.3 Evaluation of experimental methods and limitations

This project faced different experimental challenges. Due to the limited timeline of the project, there were not enough experiments performed to optimize the *in vitro* study's design. This applies to incubation times, dosages of the bacterial extracts, and how viability of cancer cells was measured. There might be a more exact way to measure viability where one could distinguish between the metabolomic activity of the cancer cell due to increased toxicity and a cancer cell that is actively proliferating. In addition, it would be useful to measure the exact number of bacterial cells used, for example, by colony counting or using a quantitative PCR protocol, to compare different results.

This work examined the potential inhibitory effects of LaBiNIC® cell-free supernatant, but the metabolomics analysis was done on the whole LaBiNIC® bacterial culture. This means that it was not possible to discriminate between the effect of the supernatant alone, used in the cancer cell experiments, and the effect of whole cells, whose metabolomic profile might differ to some extent. Regardless, postbiotics such as supernatants derived from probiotic strains have

previously been found to have the same or even more potent response in certain biological activities than live probiotics (15).

5.4 Weaknesses and strengths of the study

The research field of how metabolic products of bacterial cells might inhibit cancer cells has currently limited evidence documented in literature. This knowledge gap led to difficulties in finding an optimal study design for a pilot study. A significant challenge in the research on effects of probiotics is that studies vary greatly in experimental design, description of the methodology, and research quality in general. Many studies have been done by large food companies using a combination of probiotic strains, and the specific strains are not necessarily named correctly. Consequently, it is hard to know if their findings apply to other probiotic strains. These challenges lead to discrepancies in how probiotic strains behave in different settings (5). In this study, having a multi-strain probiotic made the findings more convoluted. It is hard to know whether the lack of inhibition resulted from the different strains neutralizing each other's effects or if the strains do not possess cancer-inhibitory mechanisms.

Regarding neuroblastoma cells and probiotics, there are currently no known studies investigating the topic, which led to numerous speculations in the discussion part. However, all the assumptions merely suggest possible mechanisms and need more exploration.

Strengths with the study was that there were many controls in the *in vitro* experiments and the availability of the LC-MS method which is highly sensitive for detecting metabolites.

5.5 Future aspect

Additional experiments are required to evaluate the properties of individual strains, determine correct dosages, and determine whether a mix of probiotic bacteria performs better concerning potential anti-cancer properties than single strains. It would be useful to analyze the metabolites in the supernatants and conduct targeted metabolomic analysis to investigate which SCFAs these bacteria produce. Doing these additional experiments would provide valuable insights for future research.

6 Conclusion

The potential of using probiotics in augmenting cancer treatment and reducing chemotherapy side effects is still very preliminary (15). More research is needed to show which strains of

probiotic bacteria are effective and under which certain conditions. In addition, there is a need to know mechanisms of action and dosages.

This pilot study attempted to determine whether probiotic product LaBiNIC® inhibits the neuroblastoma cell line Kelly and what potential bacterial metabolites could mediate that effect. The initial trial showed that Kelly was inhibited by all tested dilutions of LaBiNIC® cell-free supernatant after 24 hours, except 50% dilution. In the subsequent trial, 12.5% dilution of LaBiNIC® inhibited the viability slightly at 48 and 72 hours but not at 24 hours. Moreover, the 25% LaBiNIC® only inhibited Kelly at 72 hours in the second trial.

Lactic acid was the most abundant metabolite of LaBiNIC®. Based on reported literature, lactic acid is a potential metabolite that could mediate the inhibitory effect of LaBiNIC® towards the cancer cell line. Overall, findings of this pilot study show that further testing is needed to know whether and which probiotic strains could act inhibitory on neuroblastoma cells.

7 Works cited

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Appendix

List of metabolites from untargeted metabolomics

The LC-MS analysis was performed by Gaute Hovde Bø. List of the high confident metabolites with their normalized peak intensity value at T0 and T24. The normalization was done using dry weight of each sample as a normalization coefficient

Name	LaBiNIC® T0	LaBiNIC® T24
L-(+)-Lactic acid	142921934,5	70251849,44
GLUTAMATE	81618038,96	63033547,62
ASPARTATE	34800732,68	26818016,07
Dodecyl sulfate	24034045,61	14090489,31
3-PHOSPHOGLYCERATE	3270984,762	12364249,34
PHENYLALANINE	14000817,54	8842351,149
Elaidic acid	1499701,441	8640533,501
NORLEUCINE	11727531	8113703,31
α-D-Glucose-1,6-bisphosphate	8094871,48	5169126,742
PHOSPHOENOLPYRUVATE	2630611,059	4734561,151
ADENINE	3696171,666	4526299,71
2-Hydroxycaproic acid	13676489	4401478,425
N-ACETYLGLUTAMATE	30998304,2	4069115,449
N-ACETYLASPARTATE	48154202,07	3531462,788
4-Oxoproline	1732701,881	3501006,476
URIDINE MONOPHOSPHATE	230906,4926	3275013,705
DL-3-Aminoisobutyric acid	3713603,284	2892999,349
BETA-GLYCEROPHOSPHATE	2202673,618	2788766,835
ADENOSINE-MONOPHOSPHATE	330005,2276	2670243,962
URIDINE DIPHOSPHATE-N-ACETYLGLUCOSAMINE	2106467,252	2573193,128
Pyruvic acid	12849953,76	2422900,056
2-PHOSPHOGLYCERATE	1335666,979	2270894,907
PALMITOLEATE	550436,9462	2201733,566
3-Phenyllactic acid	4400134,082	2199038,624
BENZOATE	3607030,661	2165174,769
D-(-)-Glutamine	4835356,634	1878315,784
16-Hydroxyhexadecanoic acid	3094726,433	1636347,163
N-ACETYLSERINE	2692627,36	1380903,732
FRUCTOSE 6-PHOSPHATE	254945,9539	1380033,162
β-Alanine	1954310,341	1329587,908
OXOGLUTARATE	12589279,11	1310441,864
URIDINE DIPHOSPHATE GLUCOSE	4527605,16	1206920,963
SORBOSE	2351728,173	1129182,583
Isophthalic acid	3195374,761	1115373,016
TRYPTOPHAN	1297826,288	1052827,514

2-Hydroxyvaleric acid	2630074,459	847109,6912
CYTIDINE MONOPHOSPHATE	89970,09552	832708,8211
cis-Aconitic acid	1181414,023	674176,2458
Citraconic acid	638307,0034	582083,7553
HYDROPHENYLLACTIC ACID	2494201,872	558378,2705
ADENOSINE TRIPHOSPHATE	1972978,023	435426,9208
NP-016455	1668087,939	263537,9133
3-Hydroxy-2-methylpyridine	425674,2308	108595,9608
METHYL GALACTOSIDE	67243,42389	105325,4902
N-Acetylalanine	1241862,482	89264,87524
N-ACETYLGLYCINE	274659,3694	43411,75067