Results section

Kaare

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```
## -- Attaching packages --------------------------------------- tidyverse 1.3.2 --
## v ggplot2 3.3.6     v purrr   0.3.4
## v tibble  3.1.8     v dplyr   1.0.9
## v tidyr   1.2.0     v stringr 1.4.0
## v readr   2.1.2     v forcats 0.5.1
## -- Conflicts ------------------------------------------ tidyverse_conflicts() --
## x dplyr::filter() masks stats::filter()
## x dplyr::lag()    masks stats::lag()
##
## Attaching package: 'rstatix'
##
##
## The following object is masked from 'package:stats':
##
##     filter
```

MATRINEM - Attempts at creating a NEC mouse model

The research questions

Experiment no. 4 was the goal, but before we could test our overall hypothesis we needed to find out which combination of antibiotics and feeding scheme would results in the most NEC-like phenotype.

These research questions were investigated in 3 pre-experiments (PreExp 1-3), each with their own research questions.

Overview of all experiments

	PreExp 1	PreExp 2	PreExp 3	NEC-FvT
Aim	To test feasibility of formula feeding with PICC line			
Protocols	Testing a broad spectrum antibiotics mix in combination with formula feeding. 4 groups 1. CON-BF 2. CON-FF 3. AB-BF 4. AB-FF			

	PreExp 1	PreExp 2	PreExp 3	NEC-FvT
N	38 (32 survived)			

PreExp 1 - Feasibility Study

#Load in the data

Rationale: *Chen et al.*, showed that Maternal Antibiotics Treatment (**MAT**) resulted in mild NEC-like symptoms of mice offspring. We believed that MAT combined with formula feeding (**FF**) of offspring would result in a worsened phenotype - even more NEC-like than either treatment on it's own.

Aim: To test if it was possible to keep mice separated from the mother alive from postnatal day 3 and 48 hours onwards.

Research Questions:

Can we keep offspring alive with formula feeding? 38 mice were born to 6 different mothers. Of these 32 survived and were included in the analysis. After fine-tuning the feeding method we we able to successfully feed all surviving (32) mice every 3 hours for 2 days (48 hours).

Will MAT and FF affect levels of inflammatory cytokines in ileum tissue? 30 samples were available for analysis of concentrations of the proinflammatory cytokines INFg, IL-10, IL-12p70, IL-1b, IL-2, IL-4, IL-5, IL-6, KCGRO, TNFa. Concentrations were measured in 1-cm sections of proximal/distal ileum tissue using MSD Mesoscale Kits.

The concentrations of the various cytokines are shown in the table below:

Proinflammatory cytokines

	Total number of mice analyzed = 30				
Cytokines	**CON-BF**, N = 8[1]	**CON-FF**, N = 3[1]	**AB-BF**, N = 8[1]	**AB-FF**, N = 11[1]	**p-value**[2]
IL-10	1.16 (0.7, 1.8)	2.12 (1.2, 2.3)	1.32 (1.2, 1.6)	0.00 (0.0, 0.5)	0.012
IL-12p70	10.30 (7.4, 12.4)	14.32 (14.1, 15.8)	13.60 (10.4, 18.7)	15.96 (9.4, 17.0)	0.3
IL-1b	2.61 (2.0, 6.6)	3.10 (2.5, 9.1)	3.81 (2.8, 4.6)	2.64 (2.4, 3.9)	>0.9
IL-2	1.46 (1.3, 1.6)	1.14 (1.0, 1.3)	1.41 (1.3, 1.7)	1.22 (1.1, 1.3)	0.053
IL-4	0.72 (0.6, 1.0)	0.77 (0.7, 0.9)	0.98 (0.9, 1.1)	0.52 (0.5, 0.6)	0.004
IL-5	1.72 (1.6, 2.1)	1.34 (1.2, 1.7)	2.01 (1.5, 2.2)	0.89 (0.6, 1.1)	<0.001
IL-6	12.34 (10.8, 16.7)	39.42 (32.2, 40.6)	14.97 (14.1, 17.9)	24.06 (21.8, 30.2)	0.002
KCGRO	8.09 (4.5, 25.1)	8.91 (7.9, 17.6)	7.76 (5.7, 10.4)	7.20 (6.0, 9.4)	0.9
TNFa	2.98 (2.2, 3.7)	1.84 (1.5, 2.4)	2.64 (2.4, 3.3)	1.45 (1.4, 1.8)	0.004

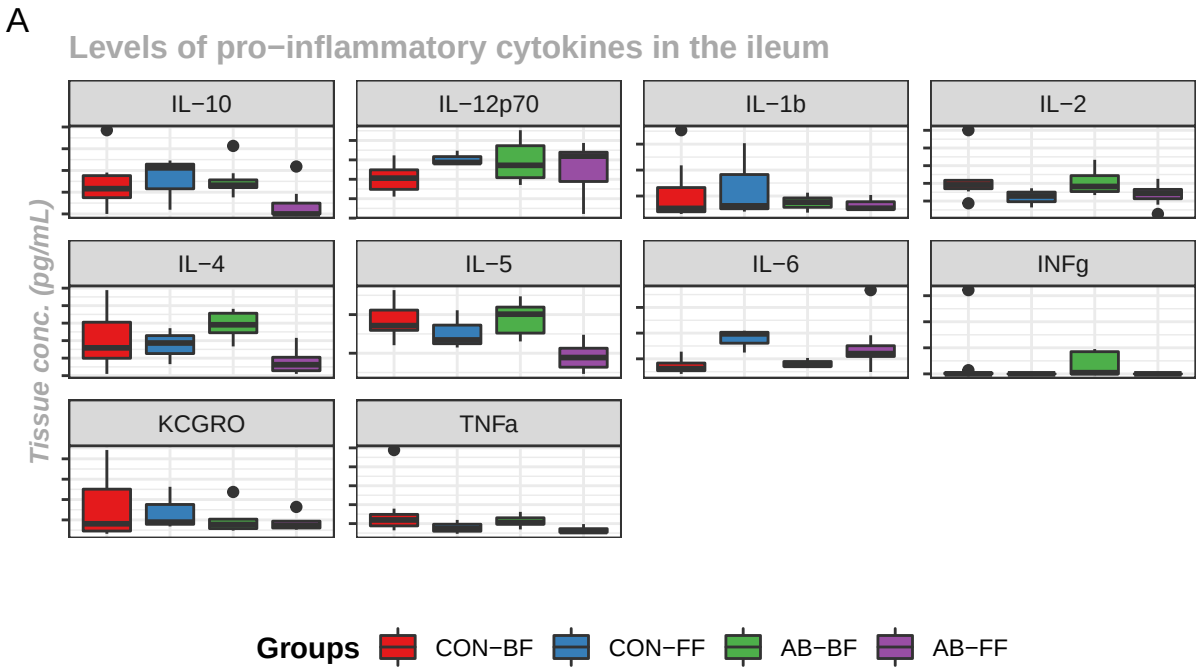
[1]Median (IQR)
[2]Kruskal-Wallis rank sum test
* P-value indicates overall group differences

Matrinem round 1

Cytokine levels are shown below:

Cytokine expression

All cytokines measured



Distributions were assessed for normality with Shapiro-Wilk Normality Test (see table below), and plot of data were made to assess distributions visually:

Table 3: Normallity check of cytokine distribution (Shapiro-Wilk Normality Test, $p < 0.05$)

cytokines	p
IL-10	0.0070630
IL-12p70	0.9452073
IL-1b	0.0000002
IL-2	0.0007820
IL-4	0.0565021
IL-5	0.5463809
IL-6	0.0000502
INFg	0.0000000
KCGRO	0.0000019
TNFa	0.0000000

Most measured cytokines wore not normally disrtibuted. Consider logtransformation

The cytokines that were significantly different between groups are shown below:

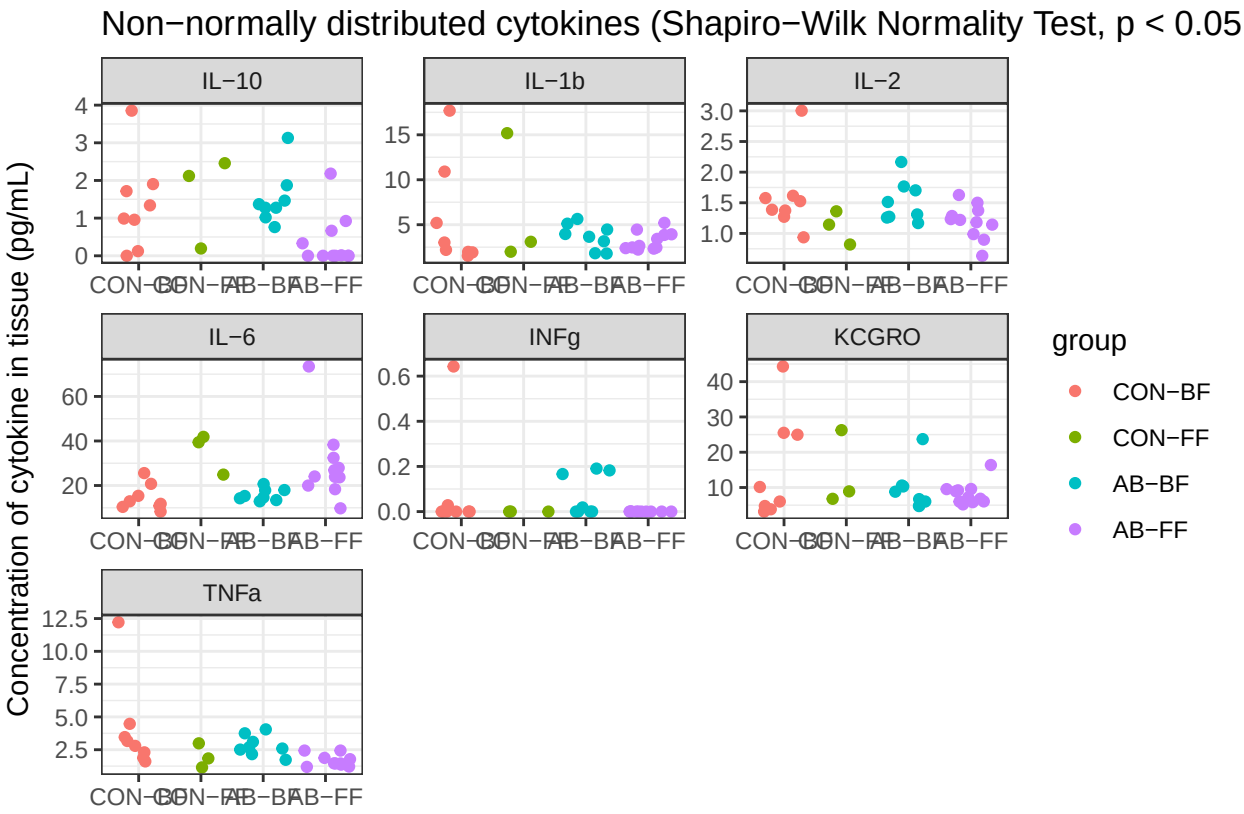
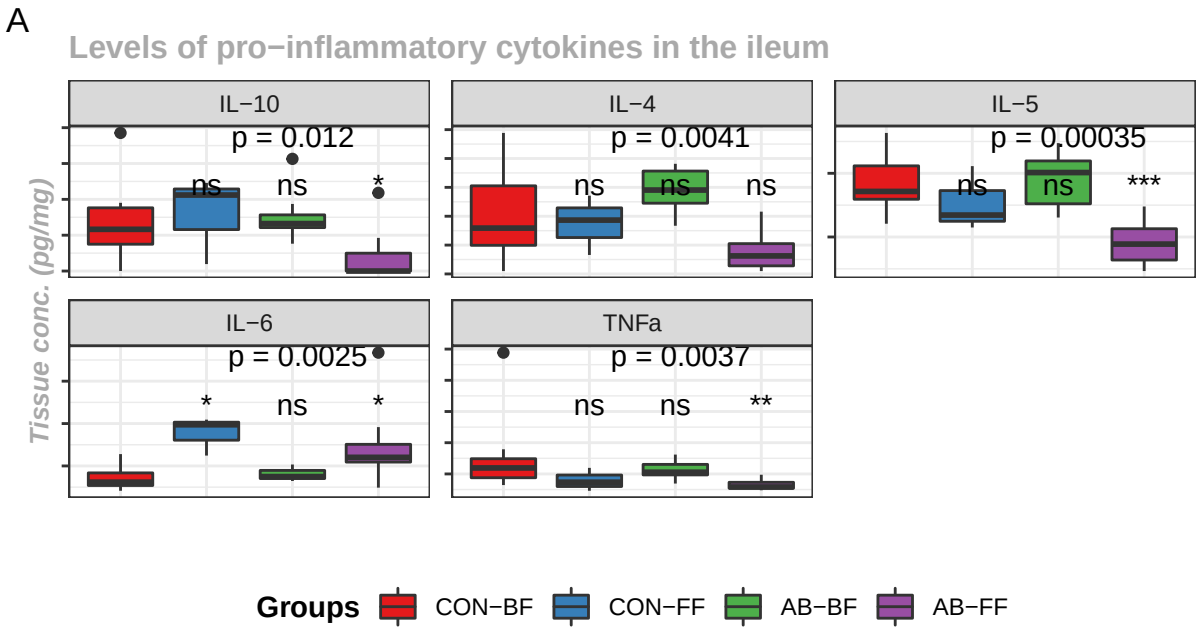


Figure 1: Non-normally distributed plots

Cytokine expression

Statistically significant changes



CON = Water, AB = Antibiotics, BF = Breast−Feeding, FF = Formula−Feeding.

Pairwise comparisons with CON−BF using wilcoxon test

The plot above shows that the cytokines significantly affected / difference were IL-10, IL-4, IL-5, IL-6, TNFa. A linear regression analysis calculates the factors responsible for the changes observed.

Table 4: Significant effectors on cytokine expression

cytokines	term	estimate	std.error	p.value
IL-10	type_of_feedFF	-1.14745	0.42366	0.01180
IL-4	type_of_feedFF	-0.43279	0.09941	0.00019
IL-4	maternal_treatmentCON:type_of_feedFF	0.36122	0.17567	0.04993
IL-5	type_of_feedFF	-0.97647	0.19223	0.00003
IL-6	type_of_feedFF	13.09137	5.14270	0.01718

Formula-feeding looks to be the most determining factor for changes to cytokine expression.

The conclusion is that many of the cytokines show an interaction effect of the two treatment parameters.

Summary of PreExp 1

- We could keep them alive with proper feeding technique.
- Formula feeding looks to be the most determining factor for IL-10, IL-4, IL-5, IL-6 (p-values 0.0117961, 1.8502541×10^{-4}, 2.723642×10^{-5}, 0.0171812, respectively).

PreExp 2 - Selectively targeting gram+ or gram- bacteria

Rationale

- Based on the results from Preexp 1 we knew that we could perform the experimental setup without too many casualties.
- We had seen that formula-feeding did affect the expression of cytokines, but visual inspection of the intestines showed that we had not managed to create a NEC-like phenotype.
- After considering the results we realized that our assumptions were based on a dysbiotics microbiome in the mother leading to less immunizing milk. However, we had likely not created a dysbiosis as much as an overall depletion by administering a broad-spectered antibiotic. We therefore hypothezised that targeting specific stains of bacteria would lead to a more dysbiotic microbiome and allow for greater disbiosis in the offspring.
- In **PreExp 2** we therefore tested whether selectively targeting gram+ or gram- bacteria would lead to different and more pronounced NEC-like symptoms.

Research questions:

Will selectively targering gram+ or gram- bacteria with Vancomycin and Gentamicin respectively lead to more pronounced NEC-like symptoms compared with no treatment on the background of breastfeeding.

Experiment 4 (NEC-FvT)

The PreExps are now over and it is time to conduct the study. Having determined the optimal experimental protocol in PreExp 1-3 we are ready with the design.

Overview - Experimental Setup

Intestinal permeability - FITC Intestinal permeability is assessed with the FITC-Dextran assay. First I inspect the distribution of results.

```
## # A tibble: 3 x 4
##   group    variable statistic         p
##   <chr>    <chr>        <dbl>     <dbl>
## 1 CON-BF   values       0.850 0.0749
## 2 FVT-FORM values       0.711 0.00190
## 3 SM-FORM  values       0.538 0.0000296
```

As results and highly skewed I try transforming the FITC results to log10.

```
## # A tibble: 3 x 4
##   group    variable      statistic       p
##   <chr>    <chr>             <dbl>   <dbl>
## 1 CON-BF   log_10_values     0.843 0.0624
## 2 FVT-FORM log_10_values     0.815 0.0304
## 3 SM-FORM  log_10_values     0.690 0.00174
```