Using Mechanistic Data to Predict and Interpret Clinical Outcomes in Probiotic Studies on Immunity.

Milano, Italy
October 16, 2015

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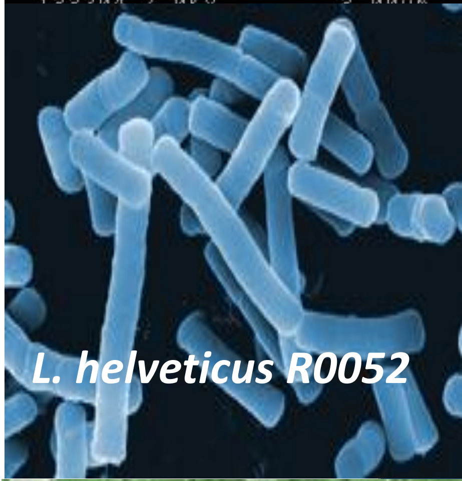
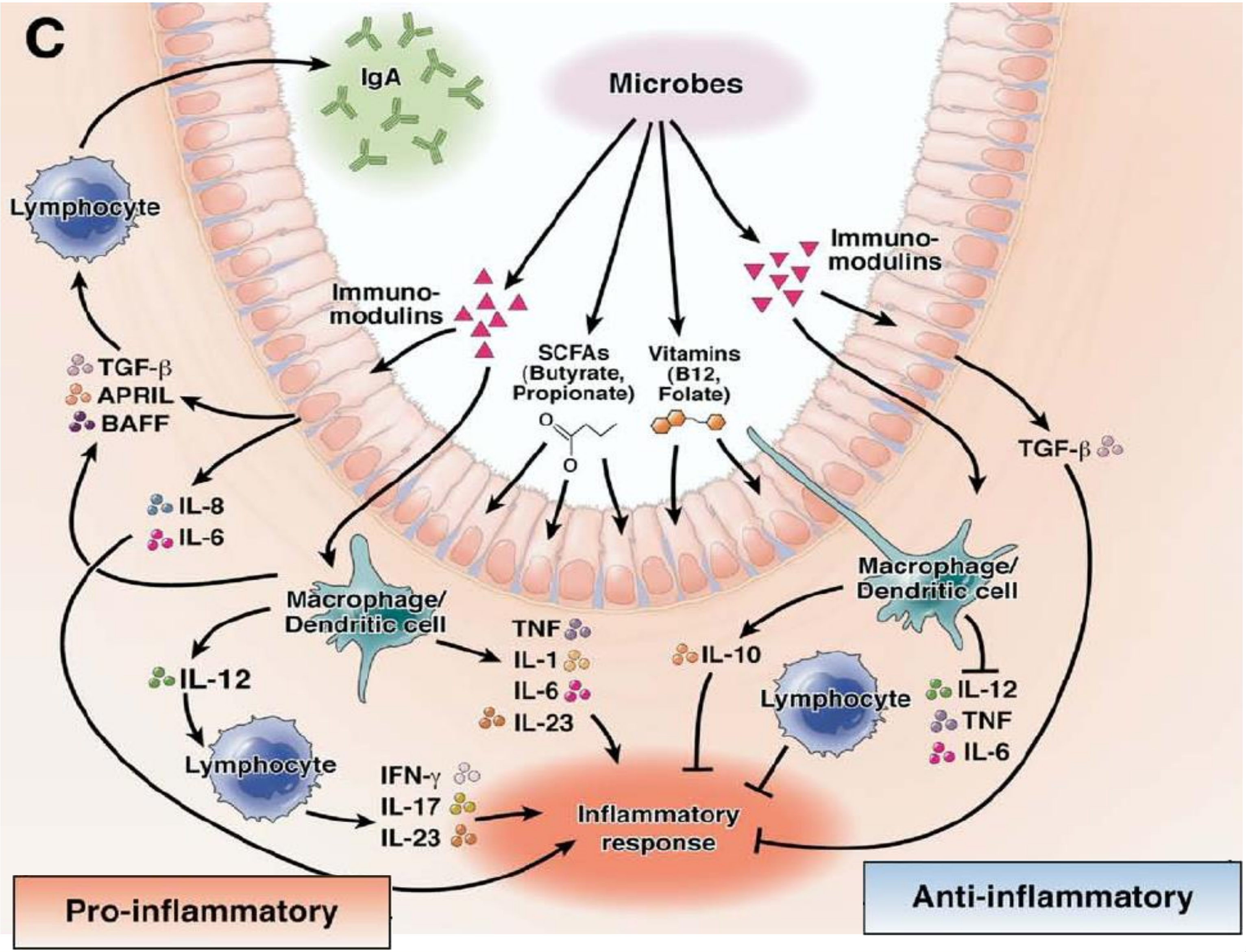
Take Away Message:

- This is a story about our attempt to integrate our pre-clinical findings with our clinical outcomes to make better choices for subsequent studies.
- Transversal pipeline development is more important than the individual results.
- This is an adaptive design and as such it is always an on-going work in progress.



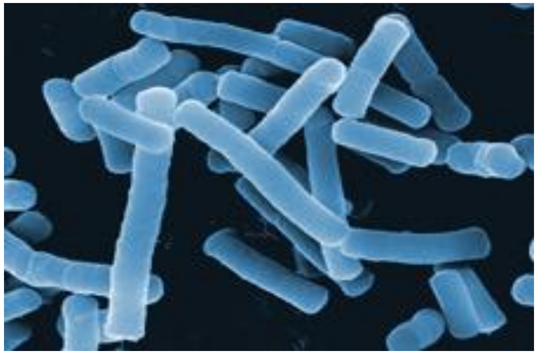
Background on Host-Microbe Interactions



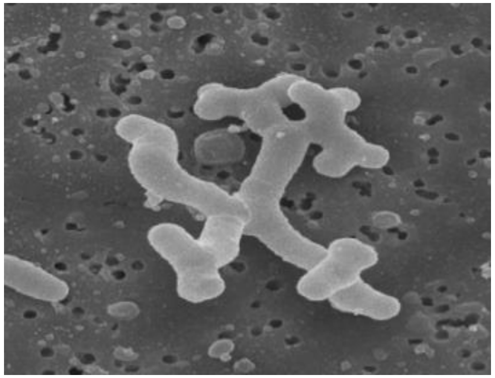
Preidis,G.A.and Versalovic, J.(2009).Gastronenterology 136:2015-2031

Multi-strain Probiotic: ProbioKid

R0052, R0033, R0071:
60:20:20 + prebiotic FOS



Lactobacillus helveticus **R0052**



Bifidobacterium bifidum **R0071**

Bifidobacterium longum **subsp.** ***infantis*** **R0033**

Previous Work:

- **Based on the positive beneficial effects on immunity with ProbioKid**

  - *Animal studies:* *Cazzola et al. 2010* (both T_H1 and T_H2 models)

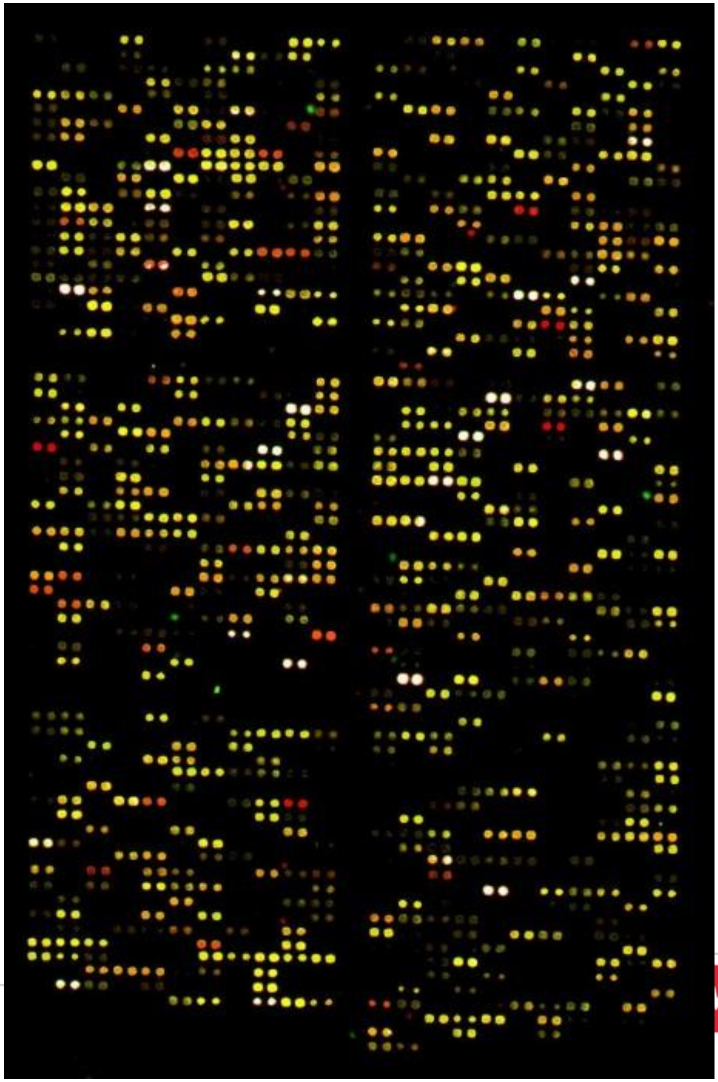
  - *Clinical:* *winter respiratory infections - Cazzola et al. 2010 & rotaviral infections - Mei & Chen, 2008*

- **Aim: To elucidate which immune pathways does this probiotic modulate in the host cell that has been stimulated by a dsRNA viral mimic, Polyinosinic:polycytidylic acid**
  - *Using in-vitro IEC cell model and viral mimic, poly (I:C)*
  - *Using a custom-designed Immune Array*
  - *Better understanding of the mechanisms of action*

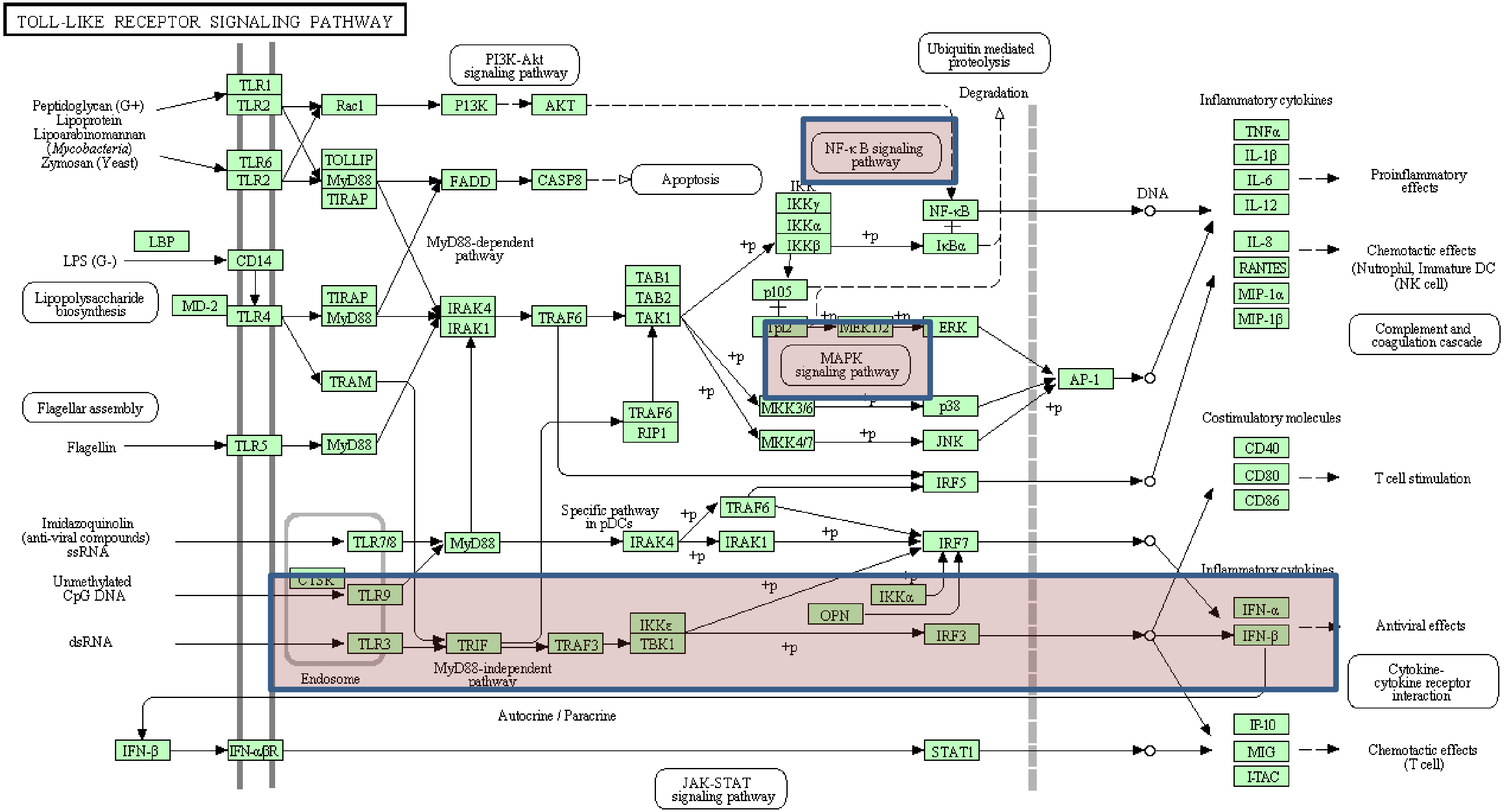
How do we evaluate this interactions:

Custom-Designed ImmuneArray: 1,354 human immune-related genes associated with innate immunity and barrier defense

1. B-Cell Receptor Signalling Pathway
2. T-Cell Receptor Signalling Pathway
3. Toll-Like Receptor Signalling Pathway
4. MAP Kinase Signalling Pathway
5. TGF-β Signalling Pathway
6. Jak-STAT Signalling Pathway
7. NF-kB Signalling Pathway
8. TNF-α Signalling Pathway
9. Epithelial Cell Signalling
10. Cytokine-Cytokine Receptor Interaction
11. Natural Killer Mediated Cytotoxicity
12. Cell Adhesion Molecules
13. Mucus Production
14. Tight Junctions
15. Gap Junctions
16. Adherens
17. Apoptosis

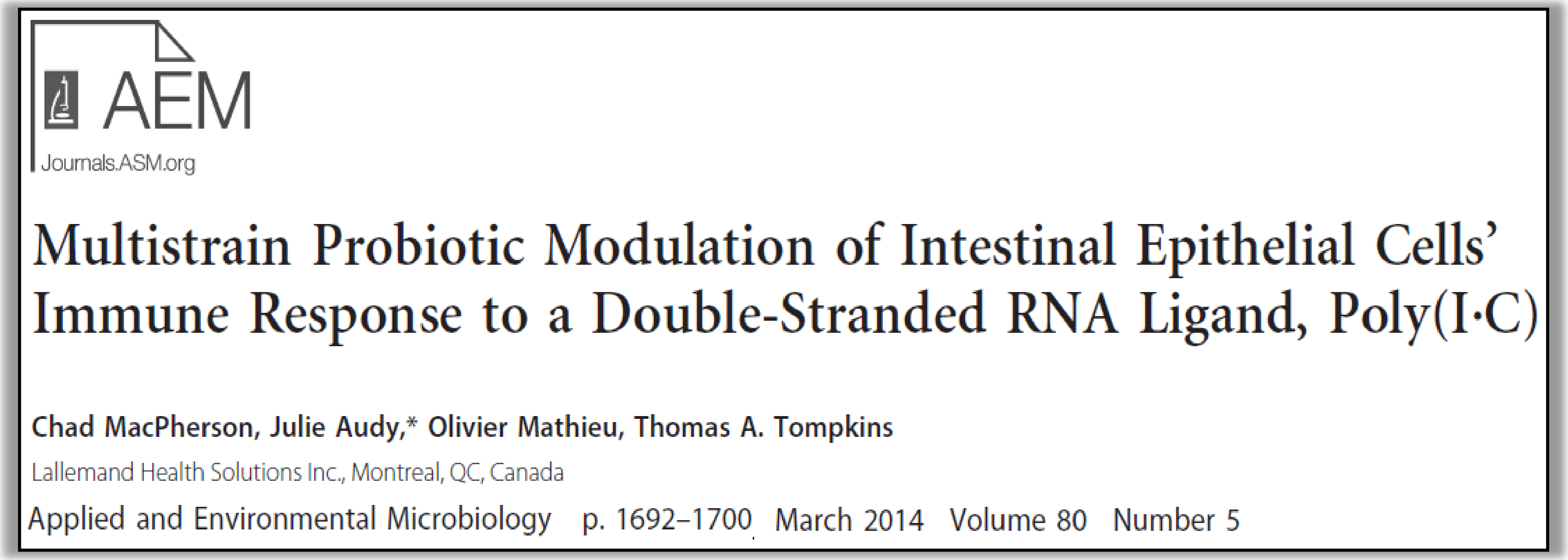


Evaluate Entire Pathways Not Just Genes



Kyoto Encyclopedia of Genes and Genomes (KEGG)

Previous Work:



AEM

Journals.ASM.org

Multistrain Probiotic Modulation of Intestinal Epithelial Cells' Immune Response to a Double-Stranded RNA Ligand, Poly(I·C)

Chad MacPherson, Julie Audy,* Olivier Mathieu, Thomas A. Tompkins

Lallemand Health Solutions Inc., Montreal, QC, Canada

Applied and Environmental Microbiology p. 1692–1700 March 2014 Volume 80 Number 5

Summary Findings AEM Publication:

(1) Overall, multi-strain probiotic (ProbioKid) had major impact at attenuating or turning off genes connected to the pro-inflammatory response induced by viral mimic, poly (I:C) challenge

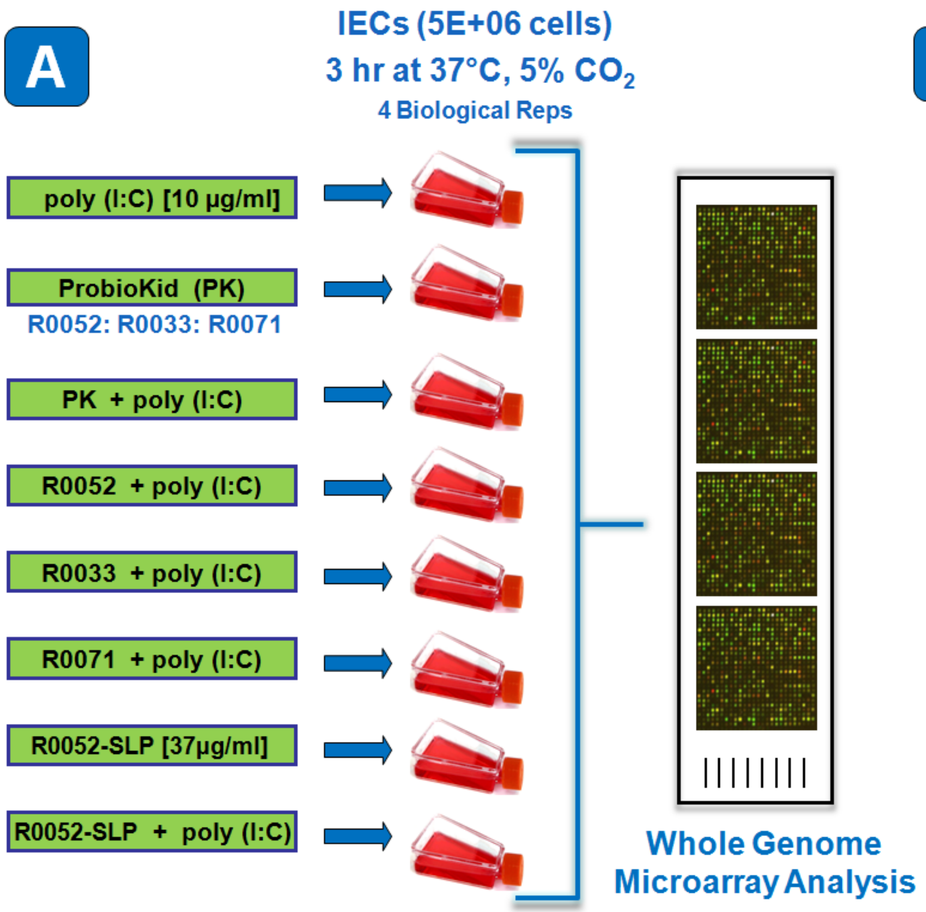
(2) Major immune pathways by which ProbioKid may be eliciting its modulatory effect included TLR3-TRIF, MAPK and NF-κB signaling pathways

Rational for Current Work:

- **A recent clinical study (*Langkamp-Henken et al.* 2015) examined the effect of the same 3 probiotic strains on the proportion of healthy days in academically stressed students**
  - ***B. bifidum* R0071 – positive health benefit;**
    - **50% ↓ # sick days;**
    - **↓ Diarrhea events**

- **Continuing the work it was decided that the following would be evaluated:**
  - ***Individual strains R0033, R0052 and R0071***
  - ***Purified S-layer protein from R0052***
  - ***Also use whole human genome microarrays from Agilent Technologies to evaluate other cellular pathways beyond immune pathways***

Experimental Design:



A

IECs (5E+06 cells)
3 hr at 37°C, 5% CO_2
4 Biological Reps

poly (I:C) [10 µg/ml]

ProbioKid (PK)
R0052: R0033: R0071

PK + poly (I:C)

R0052 + poly (I:C)

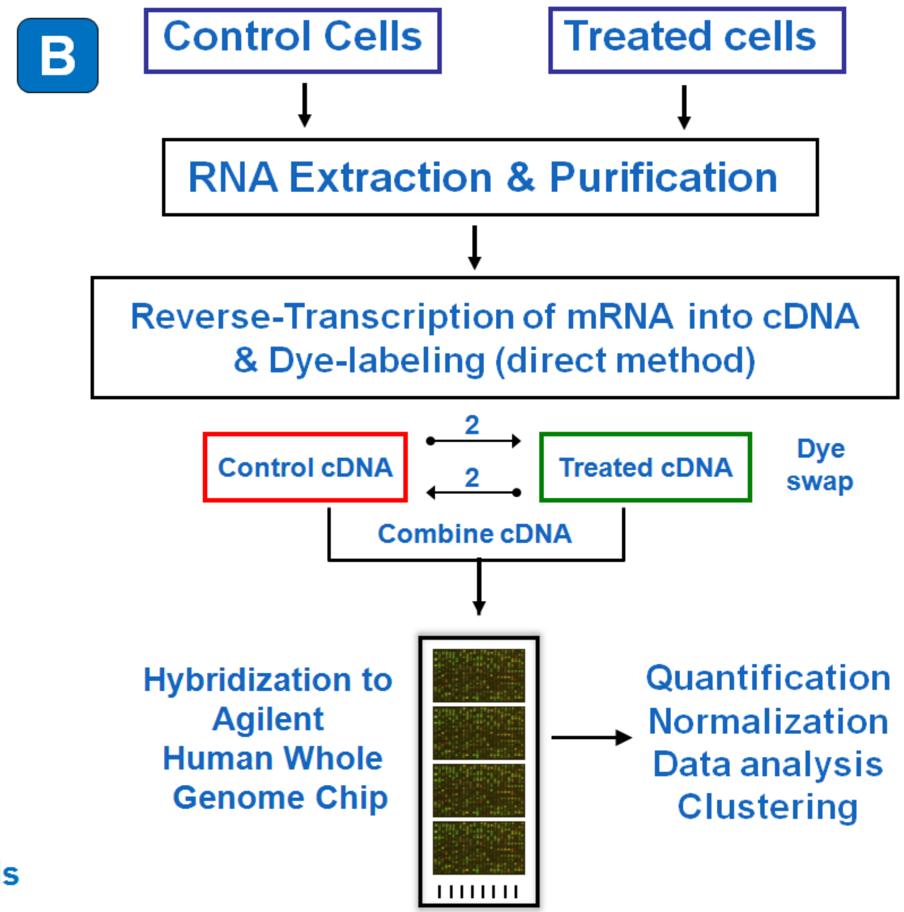
R0033 + poly (I:C)

R0071 + poly (I:C)

R0052-SLP [37µg/ml]

R0052-SLP + poly (I:C)

Whole Genome Microarray Analysis



B

Control Cells

Treated cells

RNA Extraction & Purification

Reverse-Transcription of mRNA into cDNA & Dye-labeling (direct method)

Control cDNA

2

2

Treated cDNA

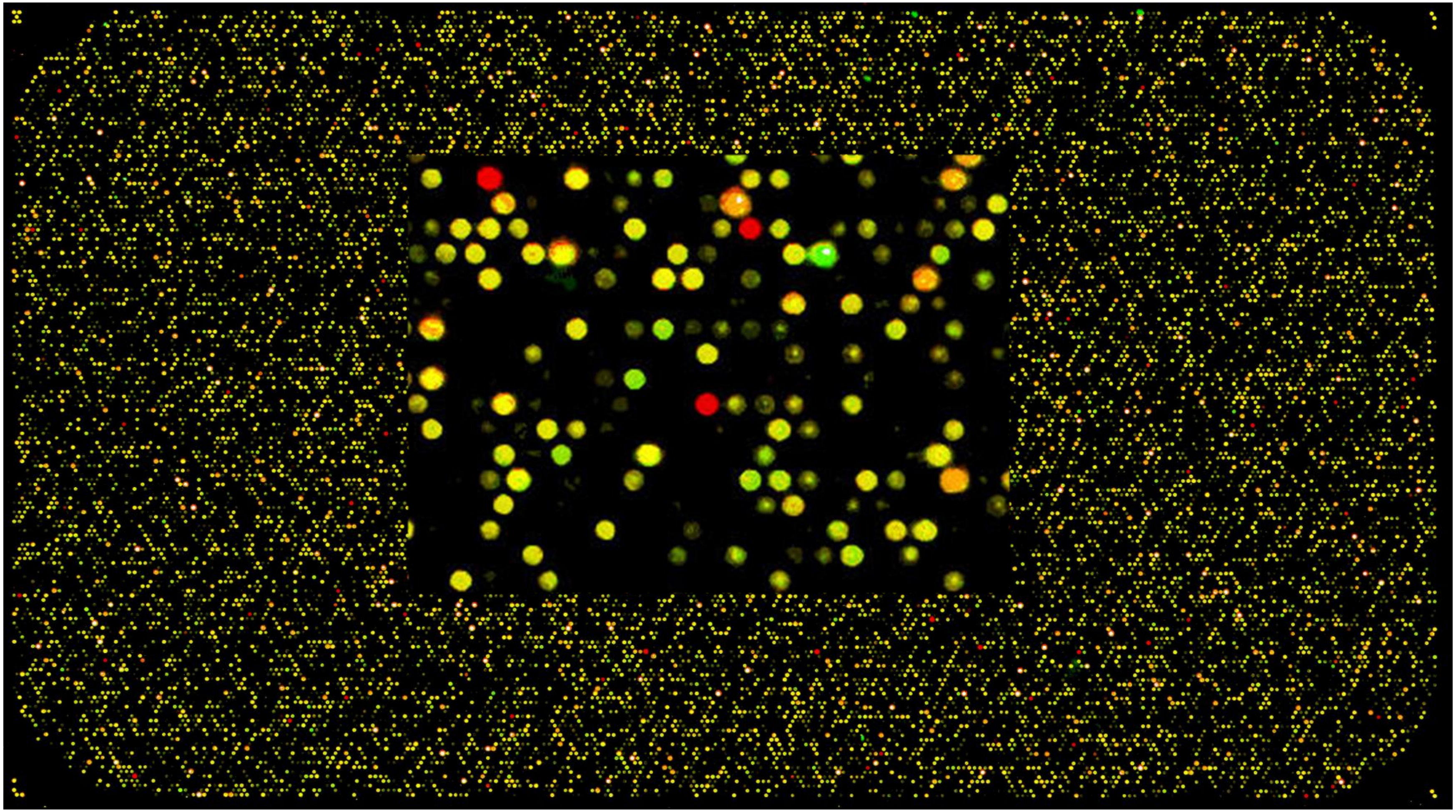
Dye swap

Combine cDNA

Hybridization to Agilent Human Whole Genome Chip

Quantification
Normalization
Data analysis
Clustering

Experimental Design: Whole Genome Microarrays

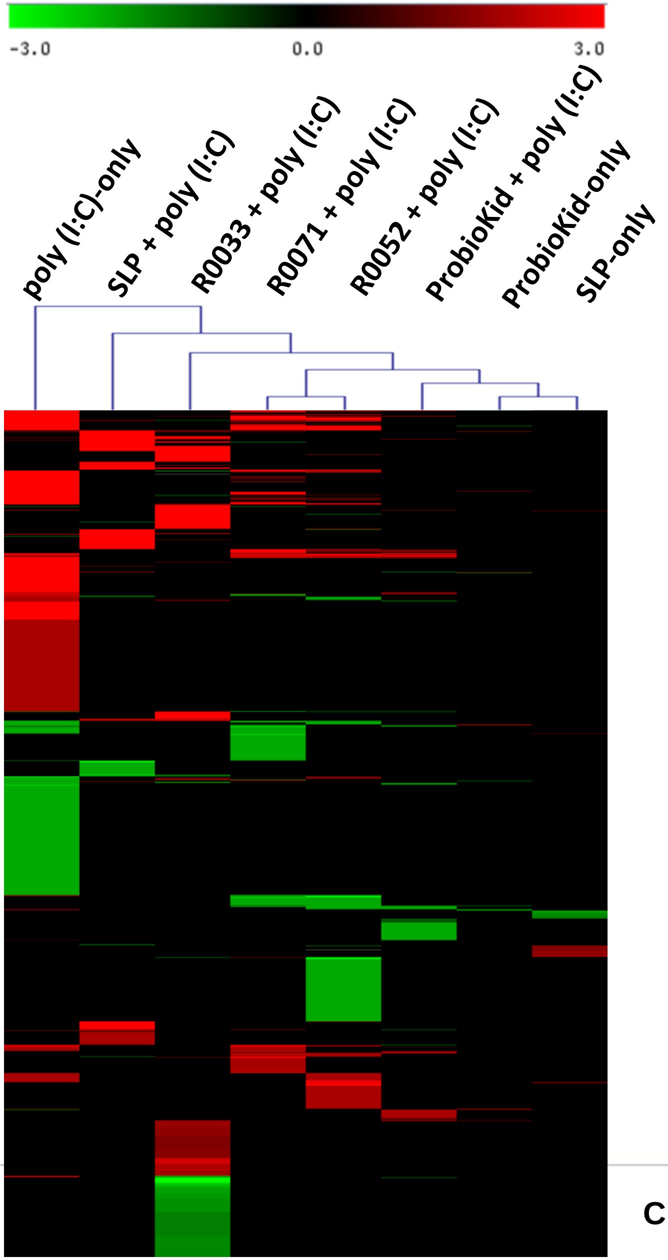


27,958 Entrez Gene RNAs
12 x 10 Control Probes
10 x 32 Spike-in Control Probes

Yellow Spots: Expression Same in Control vs Treated
Red Spots: Up-regulated
Green Spots: Down-regulated
Black spots: No gene expression

Results: Whole Human Genome Microarray

Hierarchical Clustering Heat Map



Cluster analysis of global gene expression

- Red – up-regulated
- Green – down-regulated
- Black no differential expression

Total Number of Genes Modulated:

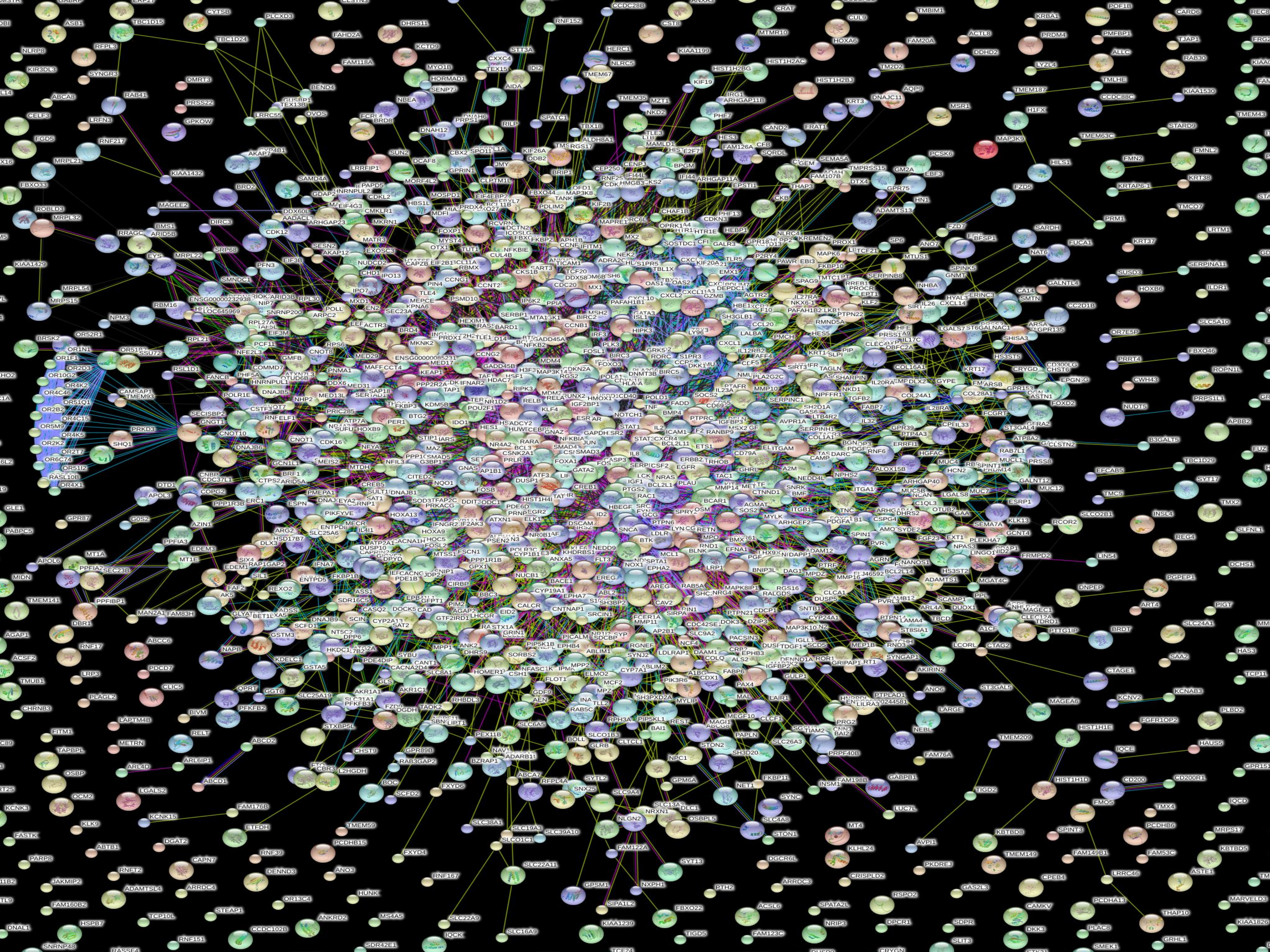
Challenges	Genes
poly (I:C)-only	863
R0033 + poly (I:C)	465
R0052 + poly (I:C)	369
R0071 + poly (I:C)	293
SLP + poly (I:C)	219
ProbioKid + poly (I:C)	132
ProbioKid-only	18
SLP-only	45

R0033, R0052, R0071 and S-layer show attenuating effect on global gene modulation as well as strain specific effects

ProbioKid has greater impact on attenuating global gene expression when challenged with poly (I:C)

ProbioKid and SLP-only had little to no effect on global gene modulation

Cut-off 1.5-fold in differential gene expression and p-value <0.05



Network labels in the dense centre include: IL2, IL8, TNF, STAT1, STAT3, EGFR, ERBB2, JUN, FOS, CREB1, SMAD3, SMAD4, NFKBIA, GAPDH, ICAM1, CXCR4, IGF1, SRC, NRAS, MMP14, BCL2L11, TAC1, CD79A, SNCA, LCK, BTK, FLT3, ANXA5, EPHA2, PTPN6, NOTCH1, CD40, HES1, KLF4, RUNX2, MDM2, MDM4, FOXO3, BIRC5, IRF3, CCL20, CXCL1, CXCL2, CXCL10, MX1, OAS1, OAS2, GZMB, TLR5, HIPK3, PLK3, GADD45A, GADD45B, CDKN2A, DNMT3B, HMGB3, NEK2, CCNB1, CCNF, CKS1B, CCNG1, CCNG2, BRCA1 (partly), BARD1, BIRC2, BIRC3, HDAC7, RELA, RELB, RIPK3, NR4A2, CSNK2A1, BCL3, RARA, PRLR, ATF3, GATA2, FOXA1, LIF, DUSP1, AP1B1, CITED2, NQO1, FOSB, EGR2, ELK1, ATXN1, HOXA9, HOXA13, PSEN2, NR0B1, CYP1B1, PTGS2, HBEGF, RAC1, PLAU, MET, SOS2, SPRY2, OSM, BCAR1, MPO, BLNK, BMX, MCL1, NOX1, EREG, AREG, BACE1, CALCR, CYP19A1, ABL2, CAV2, SH3BP2, PIN1, SHC3, LRP1, EFNA1, BNIP3L, DAG1, RGS16, MAPK8IP1, RAB5A, NRG4, FCER1A, MMP11, SIRPA, CDC42SE1, DOK3, PTPN21, CDCP1, DZIP3, AP2B1, NCF4, SLC9A2, DAAM1, LDLRAP1, ALS2, PIK3R6, CDX1, PAX4, FABP6, IGFBP2, MAL, LAIR1, MEGF10, CLCF1, BAI1, BAI2, PAPLN, STON2, SH3D20, SLC26A3, TIAM2, PRG2, CNN3, PRPF40B, PTPLAD1, NEBL, LARGE, GULP1, MAGI1, REST, PIP5KL1, RPH3A, SLCO1B3, CLTCL1, GLRB, BOLL, SNX25, SYTL2, RFPL4A, ABCA7, NPC1, GPM6A, NET1, FKBP11, ADARB1, NAV1, BZRAP1, PEX11B, SLC6A5, RAB5C, TLL2, INA, AEN, GDF9, FLOT1, CSH1, HOMER1, NFASC, IPMK, MPP2, ELMO2, MPZ, MCF2, CYP7A1, ABLIM2, SH3PXD2A, MYLIP, ABLIM1, EPHB4, PICALM, SDCBP, SYNJ2, RGNEF, CD69, SOCS2, FADD, BMP4, POLD1, PTPRC, IGFBP3, MSX2, ETS1, RHOB, A2M, SERPINC1, MMP10, CTNND1, SNRK, BMF, ITGB1, ITGA1, ARHGEF2, MYLK, AGMAT, TNC, CSPG4, PDGFA, SPIN1, PVR, AGRN, NANOS1, BCL2L14, DUSP5, CLCA1, MMP16, MPDZ, PTRF, ADAM12, DAPP1, ADAMTS1, SCAMP1, PVRL4, PTPN14, LAMA4, TBCD, SNTB1, CYP24A1, MAP3K10, IGLL1, TDGF1, SCD5, ROR1, EPHB3, CRB2, DENND1A, GRIPAP1, RND3, SYNGAP1, MEP1B, ST8SIA1, AKIRIN2, LCORL, CTAG2, ANO6, ST3GAL5, MAGEA6, DUOX1, ARL4A, MAGEC1, CLEC12A, TDRD1, PTTG1IP, A1CF, NPAS3, LINGO1, HS3ST2, MGAT4C, SEMA7A, 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KHDRBS1, EPHA7, CSNK1, KEAP1, STIP1, MAZ, NFYA, PPP1C, SMAD5, G3BP1, GNAS, CSNK2A1, SET, HUWE1, CEBPE, GNAZ, SERTAD1, IARS, NFIA, ADCY2, MAP3K1, RGS2, CUL4B, HSF1, TAF1, H3F3A, POLA1, IGF2BP1, HMOX1, ESR2, SR2

Results: Set Distiller Analysis - *Immune and Virus Related*

GeneDecks Set Distiller Analysis	Attribute Type	poly IC only	R0052 poly IC	R0071 poly IC	R0033 poly IC	R0052-SLP poly IC	ProbioKid poly IC	Probiokid only	R0052-SLP only
Immune Related					**Number of Genes Modulated**				
Immune System Phenotype	PHENOTYPE	168	85	77	87	22	8	-	-
TGF-Beta Pathway	SUPER_PATHWAY	42	10	21	-	-	-	-	-
TNF Signaling Pathway	SUPER_PATHWAY	16	12	14	-	-	-	-	-
TNF-alpha/NF-kB Signaling Pathway	SUPER_PATHWAY	12	-	-	-	-	-	-	-
Jak-STAT Signaling Pathway	SUPER_PATHWAY	11	-	-	-	-	-	-	-
MAPK Siganling Pathway	SUPER_PATHWAY	17	16	14	-	-	-	-	-
NF-KappaB Pathway	SUPER_PATHWAY	20	13	11	-	-	-	-	-
Canonical NF-kappaB Pathway	SUPER_PATHWAY	-	5	-	-	-	-	-	-
Immune response IFN Alpha/Beta Signaling Pathway	SUPER_PATHWAY	8	-	-	-	-	-	-	-
Cytokine-cytokine Receptor Interaction	PATHWAY_KEGG	19	-	8	-	7	7	-	-
Chemokine Signaling	SUPER_PATHWAY	20	14	6	-	6	6	-	-
B-cell receptor Signaling Pathway	SUPER_PATHWAY	15	-	-	-	5	4	-	-
Toll-like receptor Signaling Pathway	SUPER_PATHWAY	18	12	10	-	-	-	-	-
Monocytes	EXPRESSION	126	-	-	93	-	-	-	-
B Lymphoblasts	EXPRESSION	147	-	-	101	-	-	-	-
Dentritic Cells	EXPRESSION	114	-	-	94	-	-	-	-
T Cells (CD4+)	EXPRESSION	105	-	-	97	-	-	-	-
T Cells (CD8+)	EXPRESSION	97	-	-	92	-	-	-	-
NK Cells	EXPRESSION	105	-	-	89	-	-	-	-
B Cells	EXPRESSION	102	-	-	86	-	-	-	-
NOD-like Receptor Signaling Pathway	PATHWAY_KEGG	-	-	-	-	5	5	-	-
RIG-I-like Receptor Signaling Pathway	PATHWAY_KEGG	7	-	-	-	4	4	-	-
Natural Killer Cell Mediated Cytotoxicity	PATHWAY_KEGG	-	-	-	-	5	5	-	-
Inactivation of MAPK Activity	GO_MOLEC_FUNC	-	6	-	-	-	-	-	-
IL27-Mediated Signaling Events	SUPER_PATHWAY	-	7	-	-	-	-	-	-
BAFF in B-Cell Signaling	SUPER_PATHWAY	11	9	8	-	-	-	-	-
Virus Related									
Influenza	DISORDER	46	27	-	-	-	-	-	-
Virus infection	DISORDER	36	20	20	-	8	8	-	-
Influenza A	SUPER_PATHWAY	23	15	-	-	-	-	-	-
Interferon Signaling	SUPER_PATHWAY	18	-	-	-	-	-	-	-
Response to Virus	GO_BIOL_PROC	14	-	-	-	-	-	-	-
2,5-oligoadenylate	COMPOUND	11	-	-	-	-	-	-	-
poly(i-c)	COMPOUND	11	8	6	-	4	4	-	-
Interferon-alpha	COMPOUND	11	-	-	-	-	-	-	-
Compound/Immune Related									
Rantes	COMPOUND	25	-	15	-	-	8	-	-
Nitric Oxide	COMPOUND	57	13	36	29	36	13	-	-
H2O2	COMPOUND	48	11	29	27	26	11	-	-
Progesterone	COMPOUND	47	10	-	26	30	10	-	-
Histamine	COMPOUND	24	-	-	-	19	-	-	-

Multi-strain ProbioKid had greater impact of attenuating the immune response (synergistic)

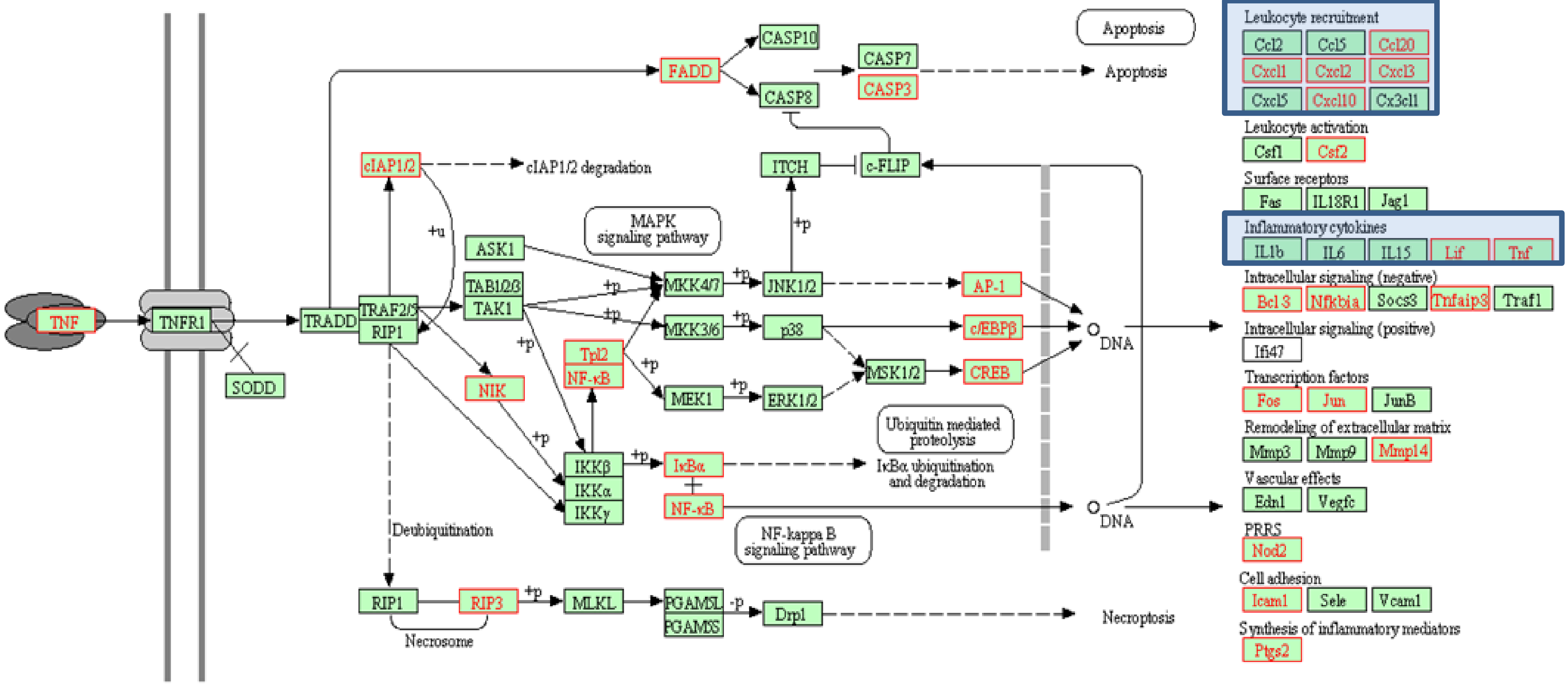
Results: Set Distiller Analysis - *Cellular, Endocrine & Disorder Related*

GeneDecks Set Distiller Analysis	Attribute Type	poly IC only	R0052 poly IC	R0071 poly IC	R0033 poly IC	R0052-SLP poly IC	ProbioKid poly IC	Probiokid only	R0052-SLP only
Cellular/Signaling Related					**Number of Genes Modulated**				
Homeostasis/Metabolism Phenotype	PHENOTYPE	196	100	80	119	26	26	-	-
Rho Family GTPases	SUPER_PATHWAY	80	36	38		-	-	-	-
Signaling by GPCR	SUPER_PATHWAY	99	-	-	59	-	-	-	-
PAK Pathway	SUPER_PATHWAY	45	-	19	-	-	-	-	-
Akt Signaling	SUPER_PATHWAY	45	27	21	-	-	-	-	-
PEDF Induced Signaling	SUPER_PATHWAY	46	-	23	-	-	-	-	-
Activation of cAMP-Dependent PKA	SUPER_PATHWAY	39	-	-	-	-	-	-	-
Focal Adhesion	SUPER_PATHWAY	23	-	12	-	-	-	-	-
Cell Adhesion	SUPER_PATHWAY	-	19	-	-	-	-	-	-
Direct p53 effectors	SUPER_PATHWAY	15	-	10	-	-	-	-	-
Wnt Signaling Pathway	SUPER_PATHWAY	16	-	12	-	-	-	-	-
Apoptosis Signaling	SUPER_PATHWAY	13	9	6	-	4	-	-	-
Integrin Pathway	SUPER_PATHWAY	38	20	17	-	-	-	-	-
EGFR1 Signaling Pathway	SUPER_PATHWAY	-	11	-	-	-	-	-	-
PI3K-Akt signaling Pathway	SUPER_PATHWAY	-	16	-		-	-	-	-
Nervous/Endocrine Related									
Nervous System Phenotype	PHENOTYPE	138	83	59	93	-	-	-	-
Behavior/Neurological Phenotype	PHENOTYPE	129	57	48	82	-	-	-	-
Endocrine/Exocrine Gland Phenotype	PHENOTYPE	85	55	33	49	-	-	-	-
Neurotrophin TRK Receptor Signaling Pathway	SUPER_PATHWAY	23	-	-	-	-	-	-	-
Disorder Related									
Necrosis	DISORDER	108	67	56	60	25	25	-	-
Inflammation	DISORDER	97	53	49	48	24	24	-	-
Inflammatory Bowel Disease	DISORDER	53	26	20	32	-	-	-	-
Crohns Disease	DISORDER	50	-	21	30	-	-	-	-
Arthritis	DISORDER	108	66	57	61	6	6	-	-
Rheumatoid Arthritis	DISORDER	94	62	53	56	8	8	-	-
Multiple Sclerosis	DISORDER	70	39	35	46	-	-	-	-
Obesity	DISORDER	71	36	-	44	-	-	-	-
Type 1 Diabetes	DISORDER	51	27	24	-	-	-	-	-
Diabetes Mellitus	DISORDER	62	-	-	40	-	-	-	-
Insulin Resistance	DISORDER	41	25	-	32	-	-	-	-
Hypertension	DISORDER	70	41	34	50	-	-	-	-
Gastritis	DISORDER	23	-	18	15	-	-	-	-
Myocardial Infarction	DISORDER	47	33	-	38	-	-	-	-

Evaluate other cellular pathways beyond the common immune pathways

Results: KEGG Pathway Analysis

TNF Signaling Pathway

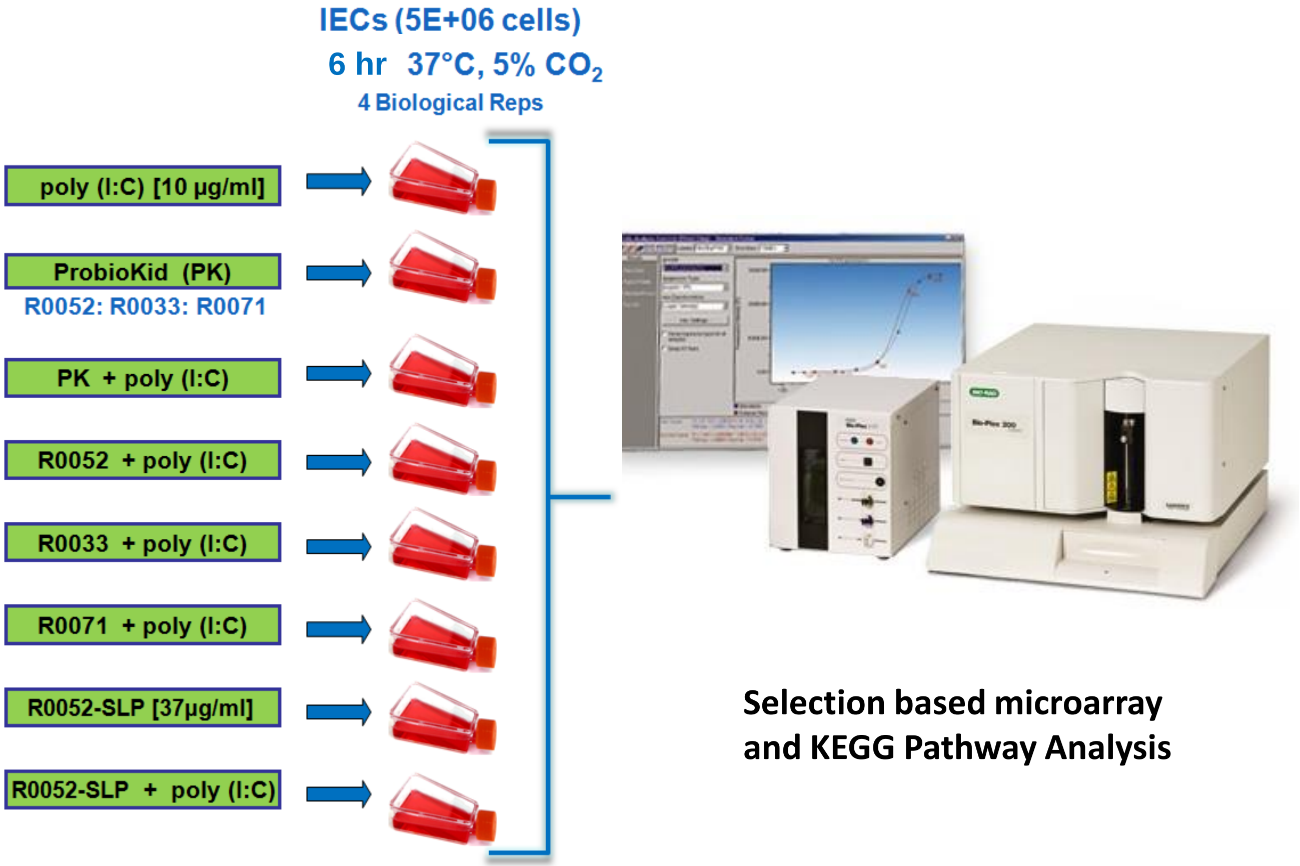


(1) Overall, have deeper analysis of what genes are modulated in a pathway
(2) Better understanding of the mechanisms of action
(3) Make a rational approach as to what markers to measure at protein level to evaluate immune responses

Kyoto Encyclopedia of Genes and Genomes (KEGG)

Cytokine/Chemokine Prolifing

In vitro BIORAD Luminex



IECs (5E+06 cells)
6 hr 37°C, 5% CO_2
4 Biological Reps

- poly (I:C) [10 µg/ml]
- ProbioKid (PK)
  R0052: R0033: R0071
- PK + poly (I:C)
- R0052 + poly (I:C)
- R0033 + poly (I:C)
- R0071 + poly (I:C)
- R0052-SLP [37µg/ml]
- R0052-SLP + poly (I:C)

Selection based microarray
and KEGG Pathway Analysis

Cytokines

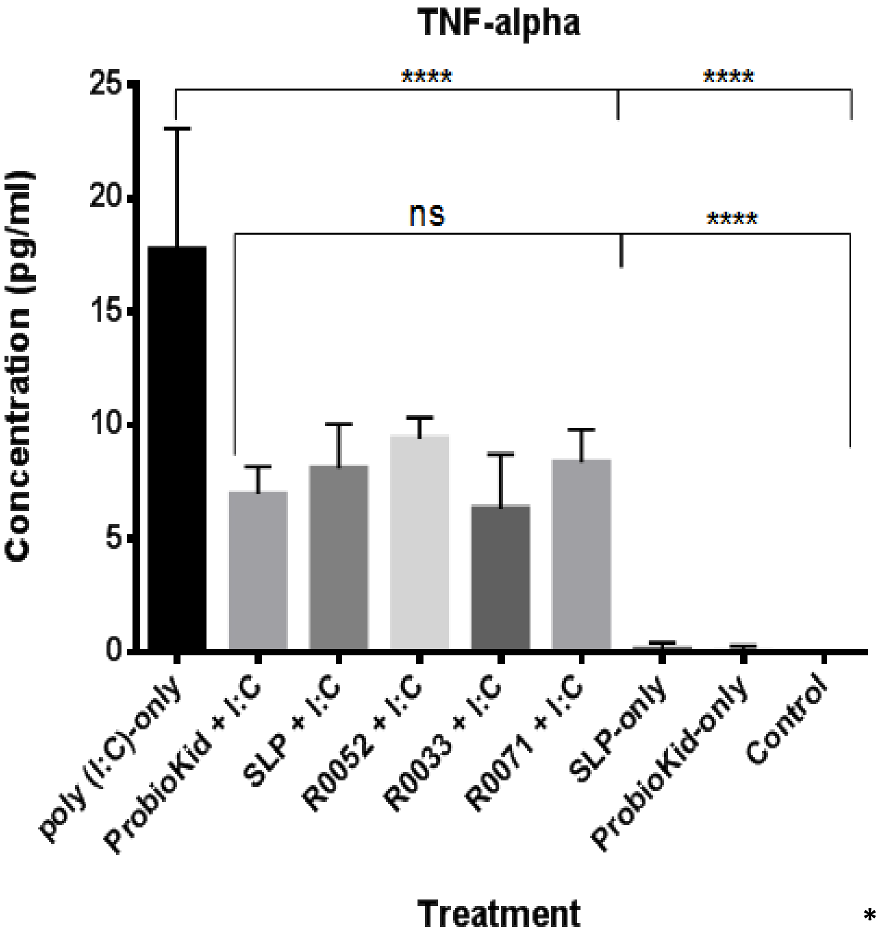
IL-1β
IL-6
IL-10
TNF-α

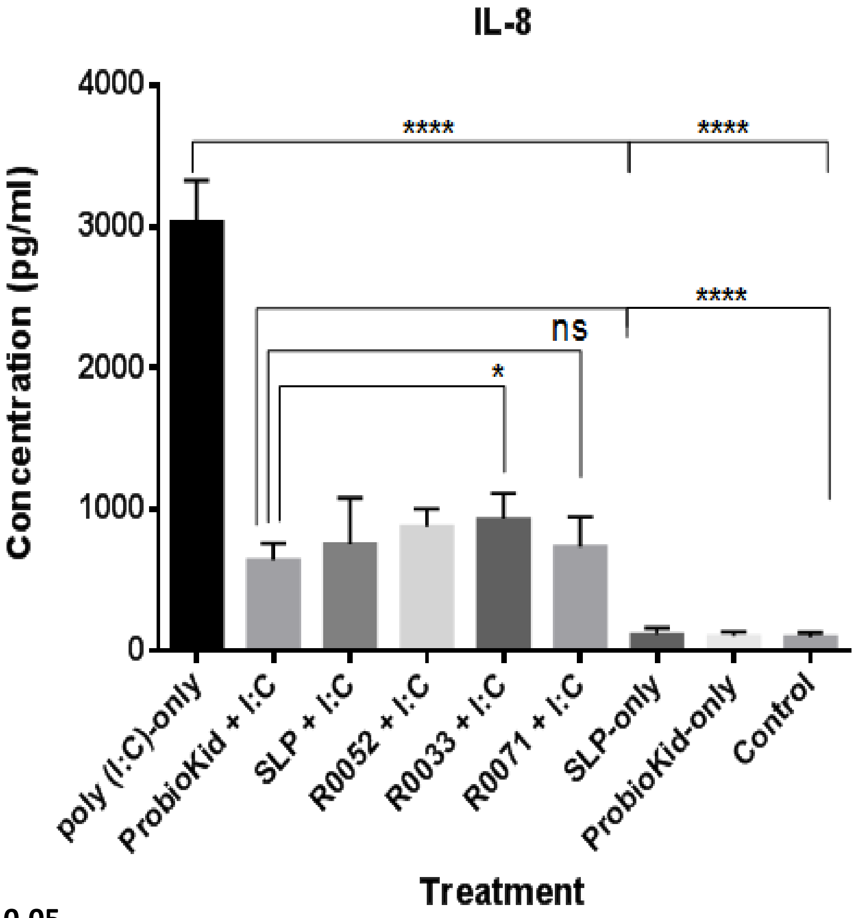
Chemokines

CXCL1
CXCL2
CXCL10
IL-8

Results: Cytokine/Chemokine Profiling

In vitro BIORAD Luminex





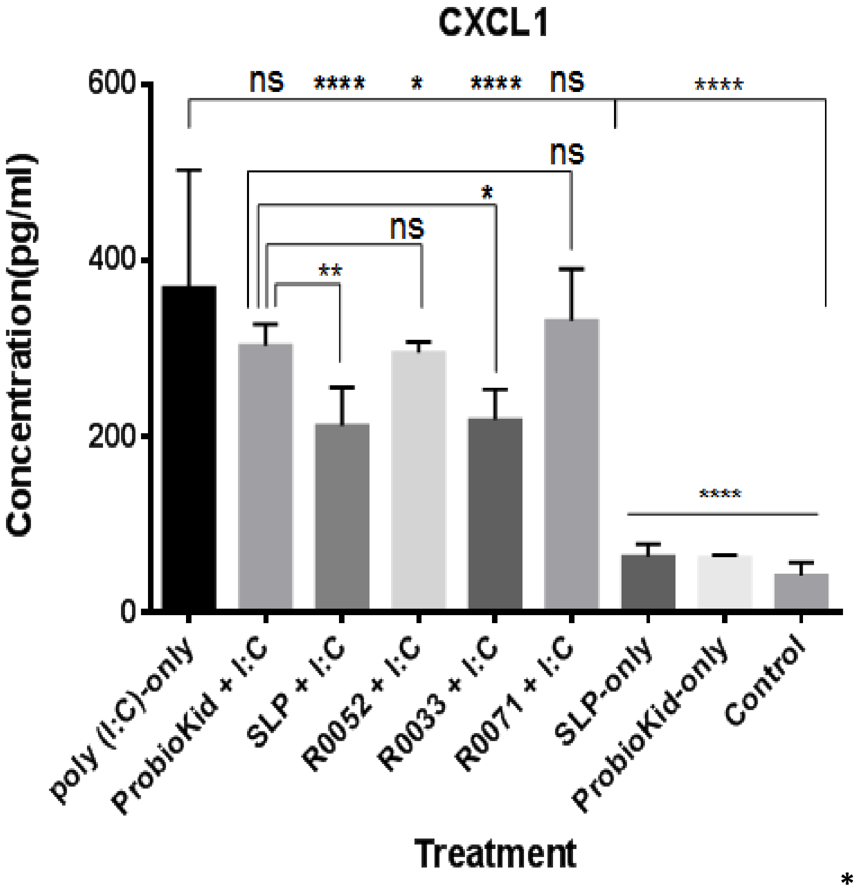
* p< 0.05
**** p< 0.0001
Two-way Anova

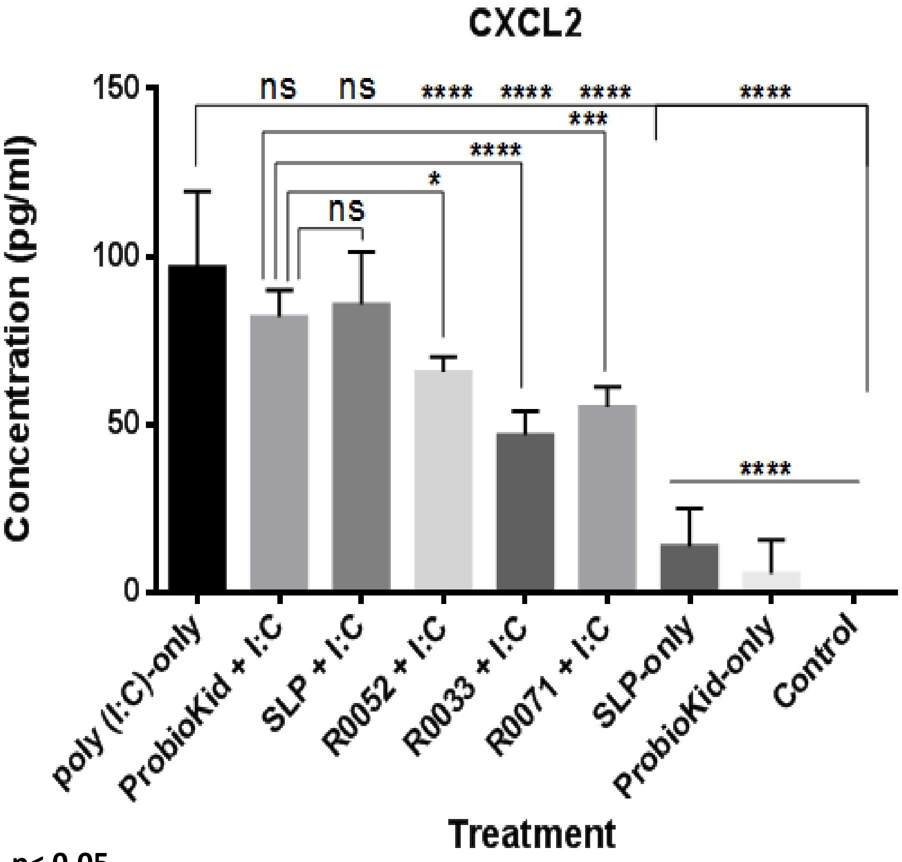
Overall, attenuation with ProbioKid, SLP, R0052, R0033 and R0071

Overall, attenuation with ProbioKid, SLP, R0052, R0033 and R0071

Results: Cytokine/Chemokine Profiling

In vitro BIORAD Luminex





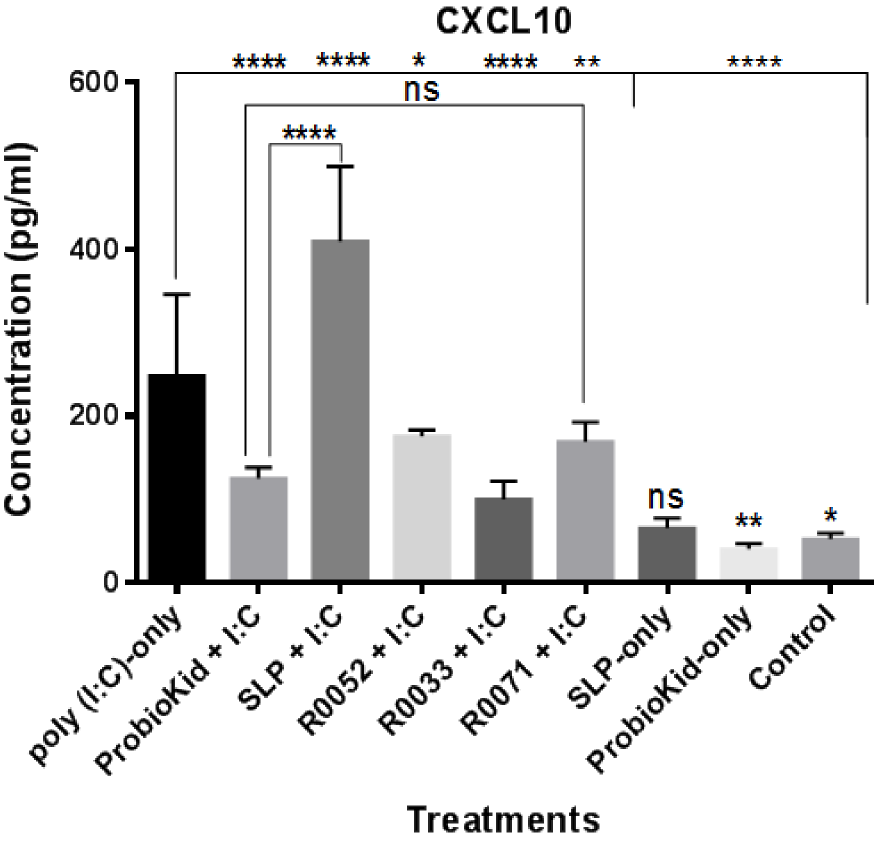
* p< 0.05
**** p< 0.0001
Two-way Anova

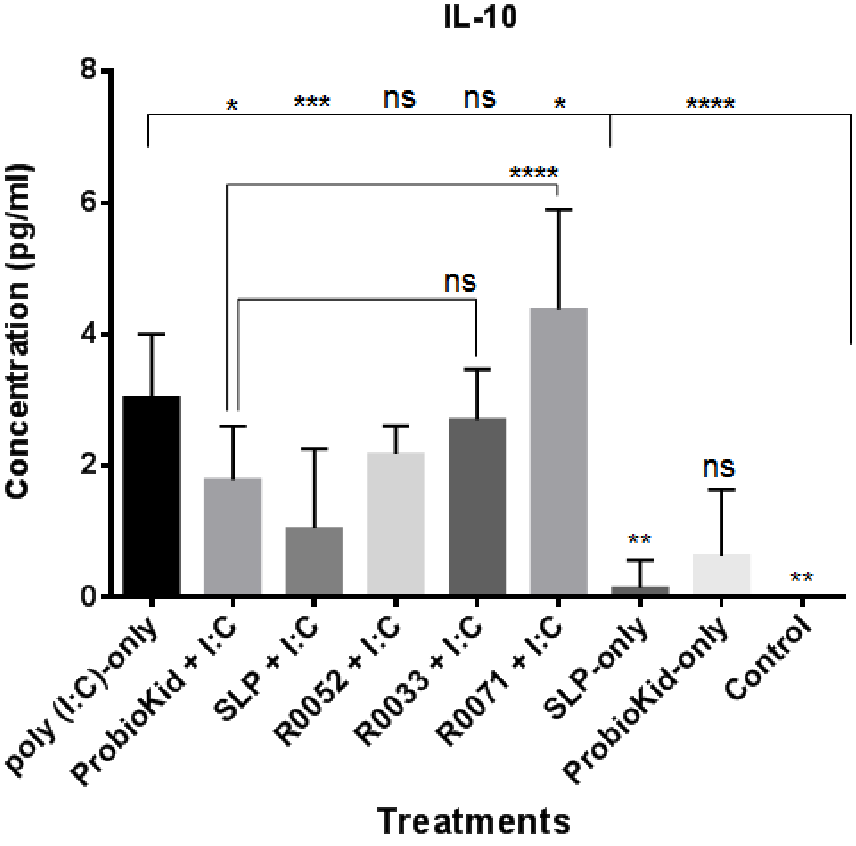
Overall, attenuation with SLP, R0052 and R0033

Overall, attenuation with R0052, R0033 and R0071

Results: Cytokine/Chemokine Profiling

In vitro BIORAD Luminex


CXCL10


IL-10

* p< 0.05
** p< 0.01
*** p< 0.001
**** p< 0.0001
Two-way Anova

Overall, attenuation with ProbioKid, R0052, R0033, R0071, but up-regulation by SLP

Overall, attenuation with ProbioKid , SLP, but up-regulation by R0071

Conclusions:

- Single probiotic strains and R0052-SLP showed strain-specific attenuation of specific gene set expression compared to the viral mimic

- The multi-strain formulation had a greater impact at attenuating global gene expression and pathway modulation compared to single strains (i.e. synergistic effect)

- Whole genome microarrays are useful because they give us a rational approach as to what markers to measure at the protein level to evaluate immune responses in clinical studies, especially in the context of the disorder or challenge.

- In particular, we believe that the T helper 17 cells may be critical control points for some probiotic strains and thus we should be looking at the cytokines: IL-17, IL-21, IL-22, and IL-23.

Future Directions:

- We are starting to understand the expression of genes/pathways modulated in intestinal epithelial cells by some of our probiotic bacteria. But this needs to be extended to other strains & products and other challenges.

- This can be applied to any human cell system: cell lines, primary grown cells, biopsies and clinical samples.

- By changing the human genome microarray to a rodent array (or use mRNA-seq), we can verify our findings in an animal model before committing to a human study.

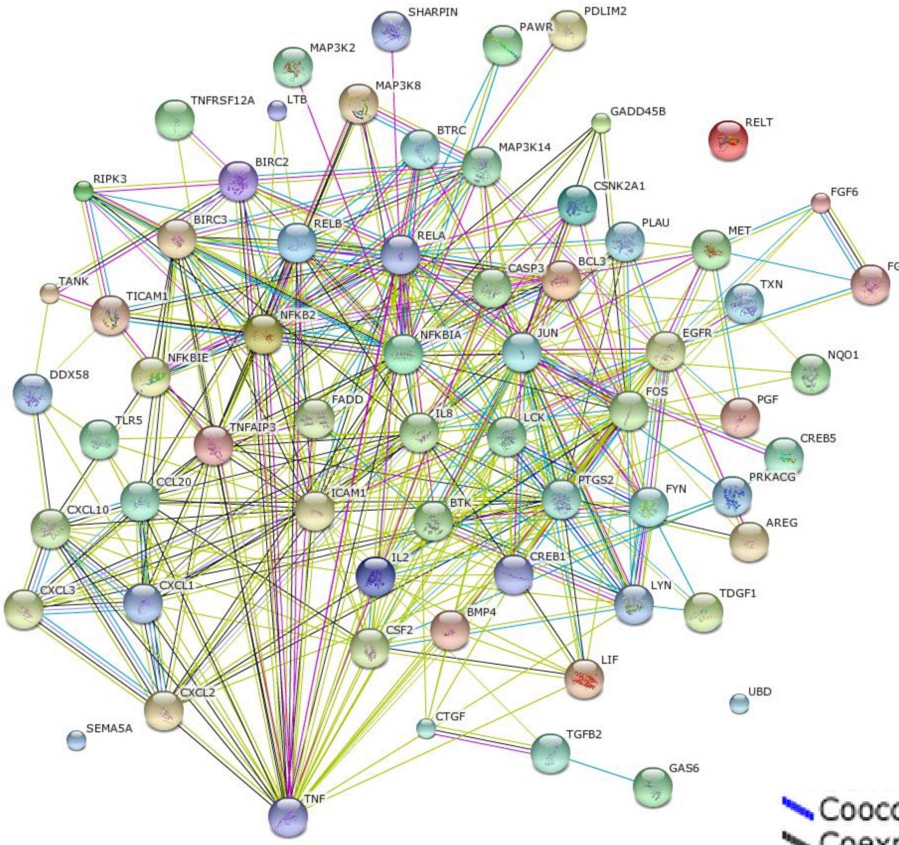
- It is a long process but highly adaptive approach.

Thanks…

Dr. Pierre Burguière, LHS
Dr. Stephanie-Anne Girard, LHS
LHS R&D team, Montreal, Canada

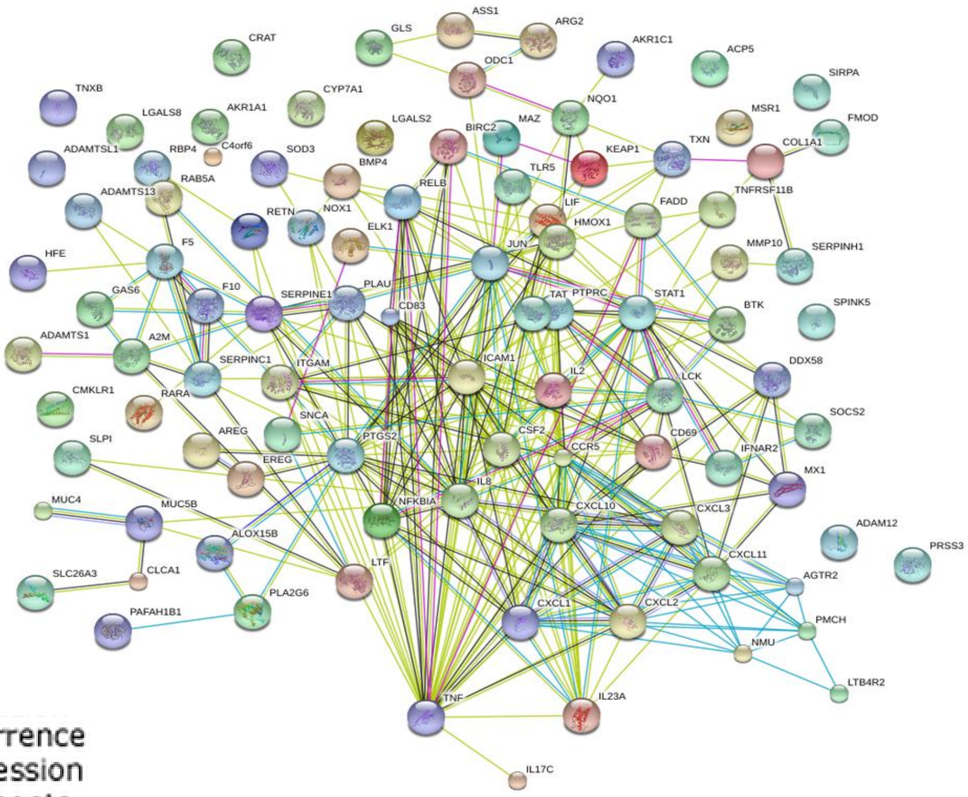
Dr. Andre Nantel - NRC, Montreal
Dr. Cecile Beaurepaire – NRC, Montreal

Gene Network Analysis Using String 9.1



TNF & NF-KB Signaling

Attribute from Set Distiller Analysis



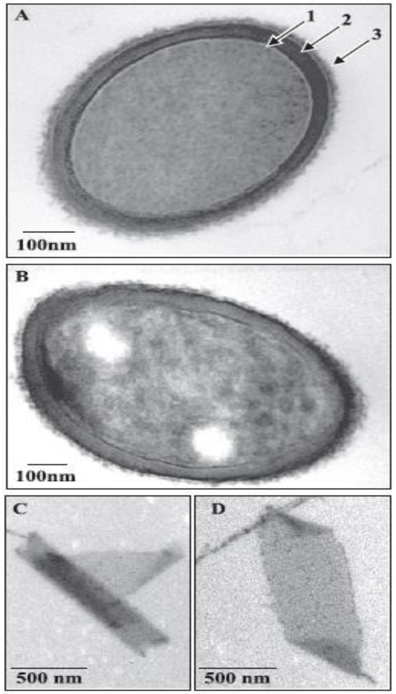
Inflammation Disorder

Attribute from Set Distiller Analysis

Experimental Design: Purity of S-layer Protein

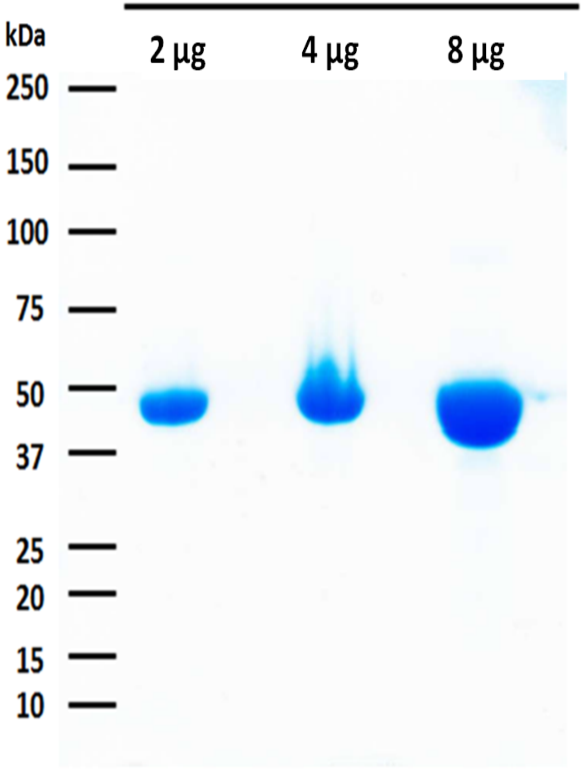
For In vitro Microarray Analysis

1-plasma membrane
2-cell wall
3-S-layer

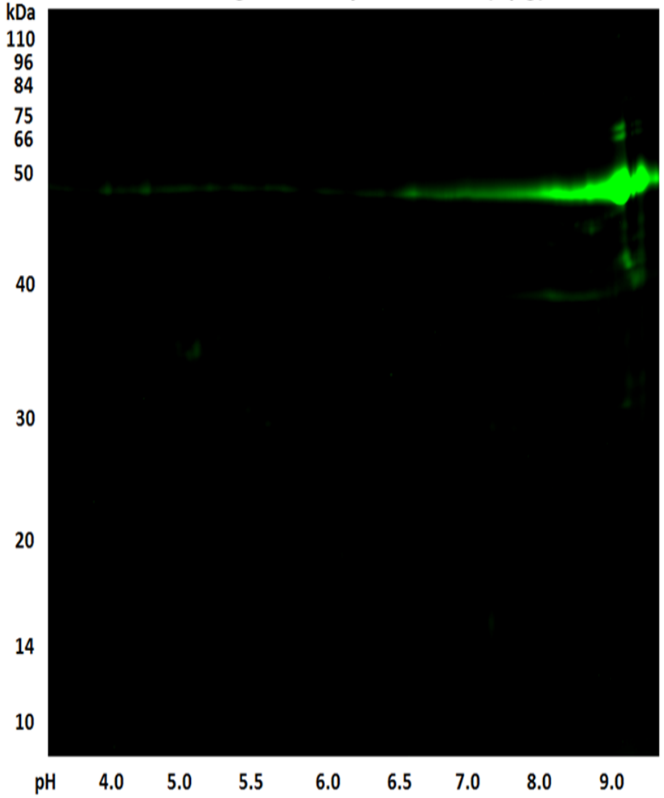


Transmission and scanning electron microscopy of *L. helveticus R0052* (Johnson-Henry *et al.*, 2007)

1D-gel of S-layer Protein



2D-gel of S-layer Protein (1µg)



Purification of S-layer protein from R0052 for microarray analysis showing high purity