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UNIVERSIDAD AUTÓNOMA METROPOLITANA

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APLICACION DE ALGORITMOS BIOINFORMÁTICOS PARA EL PROCESAMIENTO MASIVO DE DATOS PROVENIENTES DE LA SECUENCIACIÓN DEL GENOMA MICROBIANO: CARACTERIZACIÓN DE LA MICROBIOTA INTESTINAL EN MODELOS EXPERIMENTALES DE TRASTORNOS DEL NEURODESARROLLO.

En la Ciudad de México, se presentaron a las 12:00 horas del día 22 del mes de septiembre del año 2016 en la Unidad Iztapalapa de la Universidad Autónoma Metropolitana, los suscritos miembros del jurado:

DR. GUSTAVO PACHECO LOPEZ
DR. JOSE FELIX AGUIRRE GARRIDO
DR. SELVASANKAR MURUGESAN

Bajo la Presidencia del primero y con carácter de Secretario el último, se reunieron para proceder al Examen de Grado cuya denominación aparece al margen, para la obtención del grado de:

MAESTRA EN CIENCIAS (INGENIERIA BIOMEDICA)

DE: SEICY ADRIANA VELAZQUEZ VILLEGAS

y de acuerdo con el artículo 78 fracción III del Reglamento de Estudios Superiores de la Universidad Autónoma Metropolitana, los miembros del jurado resolvieron:

Aprobar

Acto continuo, el presidente del jurado comunicó a la interesada el resultado de la evaluación y, en caso aprobatorio, le fue tomada la protesta.

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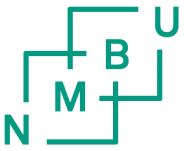

UNIVERSIDAD AUTÓNOMA METROPOLITANA

En colaboración con:

Centro de Investigación y Estudios Superiores (CINVESTAV-IPN), México
y la Universidad Noruega de Ciencias de la Vida (NMBU), Noruega.

Aplicación de algoritmos bioinformáticos para el procesamiento de datos provenientes de la secuenciación masiva del genoma microbiano: *Caracterización de la microbiota intestinal en modelos experimentales de enfermedades del neurodesarrollo.*

Tesis que presenta:
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Para obtener el grado de
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Resumen

La esquizofrenia es un trastorno mental que se caracteriza por la desintegración del proceso del pensamiento y de la capacidad de respuesta emocional. Se manifiesta más comúnmente como alucinaciones auditivas, delirios paranoides o extravagantes, o lenguaje y pensamiento desorganizado, y se acompaña por una significativa disfunción social. Se generó una infección materna utilizando un modelo de ratón por medio de la activación inmune prenatal durante el embarazo por el mimético viral poly(I:C) que redujo la sociabilidad y aumento la expresión de miedo en las crías descendencia para demostrar una posible disbiosis en la microbiota intestinal entre los ratones descendientes inmunemente activados (poly(I:C)) y los ratones descendentes control o vehículo (estériles libres de pirógenos). Se crearon dos generaciones descendencia F_1 y F_2. La secuenciación masiva del genoma microbiano se llevo a cabo utilizando dos tecnologías de secuenciación diferentes (Ion Torrent PGM e Illumina Miseq) y el análisis bioinformático se basó en el uso de herramientas tales como Qiime, USEARCH, UCHIME y UPARSE entre otras. La investigación se dividió en tres objetivos principales. En el primero se demostró el desarrollo de una disbiosis en la primer generación (F_1) entre las crías de ratones inmunemente desafiadas (poly(I:C)) y las crías de ratones control o vehículo, con ambas plataformas de secuenciación y el mismo análisis bioinformático, evaluando así las posibles similitudes y diferencias que pudieran existir en los resultados entre plataformas. Se encontraron diversos OTUs significativos entre los dos grupos tratamiento de ambos análisis, en especial ordenes de los fila *Bacteroidetes* y *Firmicutes* (*g_Prevotella, s_Barnesiella*), algunas de estas comunidades bacterianas se mantienen presentes, independientemente del tipo de

secuenciador a utilizar. En el segundo objetivo se evaluó la existencia de una posible transmisión de las comunidades bacterianas que están causando disbiosis en el grupo poly(I:C) de la F_1 a la F_2. El orden *Clostridiales*, específicamente algunas especies *Gracillibacter* que causan disbiosis en la F_1 están siendo transmitidas a la F_2 manteniendo la disbiosis entre los grupos tratamiento con una menor abundancia en el grupo poly(I:C) en ambas generaciones. El orden de *Bacteroidales* también presenta una posible transmisión de F_1 a F_2 con especies *Barnesiella* que muestran diferentes patrones de abundancia, ya que no se muestra claramente una relación entre la transmisión de estos OTUs significativos y su presencia o ausencia en el grupo poly(I:C), pero es evidente que juegan un papel importante en la disbiosis de ambas generaciones. Sin embargo, en el tercer objetivo donde se buscó un linaje (POL-M o POL-P) que fuera determinante en la transmisión de las comunidades bacterianas de la F_1 a la F_2, en el mismo cluster de *Bacteroidales* al separarse por linajes muestra una asociación de los OTUs siguiendo una dirección, se muestra una presencia o abundancia menor en los grupos poly(I:C) con respecto a los control, por parte de la F_2 la mayoría de ellos en POL-P y uno de ellos en POL-M, mostrando que el linaje paterno (POL-P) es relevante en el transmisión de comunidades bacterianas (*Bacteroidales*) de la primera a la segunda generación. Estos hallazgos apoyan la idea de una diferencia en la composición de la microbiota intestinal entre una descendencia control y una descendencia que presenta síntomas de un trastorno del neurodesarrollo (esquizofrenia), reafirmando la conexión existente entre la microbiota intestinal y el cerebro. Así como también demuestran la transmisión de grupos bacterianos causantes de disbiosis entre los grupos tratamiento de una generación F_1 a otra F_2.

1. Background

1.1 Microbiota

1.1.1 Gut Microbiota

Humans are considered host to an amount of microorganisms that are grouped in complex and usually beneficial communities, which exceed the number of human cells by, tenfold[33]. The microbiota is a complex ecosystem, which is composed of trillions of bacterial cells in the body, collectively encoding more than 3.3 million non-redundant genes; exceeding by 150 times the genome encoded by the human host[9]. These bacterial communities occupy several different but distinct microbial ecosystems on-, and within the human body, including: nasal, oral, and otic cavities, the surface of the skin, the urogenital, and the gastrointestinal (GI) tracts[45]. It is important to mention that is within the GI tract where the principal reservoir of microbes in humans is contained, of at least 1,000 different microbial species. The two most predominant bacterial communities in the GI tract are the *Bacteroidetes* (~ 48%) and *Firmicutes* (~ 51%). The remaining 1% is composed of other bacterial groups, such as *Proteobacteria, Verrucomicrobias, Fusobacteria, Cyanobacteria, Actinobacteria*, and *Spirochetes*, plus several species of fungi, protozoa, viruses, and other microorganisms[45]. Microbial colonization of the GI tract and its composition along the life of the host will depend on various factors[38]. After birth, different kinds of environmental microbes colonize the GI tract of the newborn, forming the intestinal microbiome. Infant gestational age, type of delivery, type of nutrition, and early use of antibiotics alter the composition of the microbiota and can have significant and long lasting effects[49].

The gut microbiota has several functions in the human host. For instance, it is directly involved in the synthesis of vitamins and cofactors, the breaking of complex lipids and polysaccharides, as well as on the detoxification of waste particles / toxins[40]. Through fermentation, gut microbiota produces short-chain fatty acids that play important roles working as fuel sources and maintaining the integrity of the gut epithelial attachment. At the same time, this could be related to disorders for which a "permeable gut " precedes the development of important diseases such as type1 diabetes[57]**.**

1.1.2 Microbiota – Gut – Brain axis

The microbiota-gut-brain axis, a strong and constant interaction between the gut microbiota and the central nervous system (CNS), is a set of communication channels established between the brain and the GI tract that provides to the intestinal microbiota and its metabolites a way by which interact with the brain, and vice versa. This axis includes the CNS, the neuroimmune and neuroendocrine systems, the sympathetic and parasympathetic arms of the autonomic nervous system (ANS), the enteric nervous system (ENS) and of course the intestinal microbiota[13]. These components interact to form a complex network with afferent fibers projecting to integrative structures of the CNS and efferent projections to the smooth muscle. This bidirectional communication network enables brain signals to influence the sensory, motor and secretory GI tract modalities as well as immune functions, including modulation of cytokine production by cells of the mucosal immune system, and conversely visceral messages from the gut may influence brain function, particularly brain areas involved in the regulation of stress[21].

In the case of the ENS the effector arm integrates the responses and modulates the immune activity. By the other way, the afferent limb comprises sensory nerves that contribute to intestinal reflexes and transmit information to the brain about harmful stimuli such as bowel distension and potentially dangerous signs, including the presence of bacterial endotoxins or pro-inflammatory cytokines[13].

The ANS connects the intestine with the brain. The vagus nerve is a major pathway for signals originating from the foregut and proximal colon, whereas the sacral parasympathetic nerves innervate the distal colon. The sympathetic system mainly exerts an inhibiting influence on the intestine, decreased intestinal motor function and secretion through the release of neurotransmitters such as noradrenaline[13, 21].

The humoral components of the microbiota-gut-brain axis consist of the hypothalamic-pituitary-adrenal axis, the enteroendocrine system and the immune system of the mucosa. Enteroendocrine cells from the gut epithelium produce hormones such as cholecystokinin and ghrelin, which regulate appetite, and the 5-hydroxytryptamine, which has a wide range of effects on intestinal and brain functions[13, 8].

It is known that emotional factors, stress or depression influence in the natural history of chronic GI diseases such as inflammatory bowel disease (Crohn's disease and ulcerative colitis) and inflammatory bowel syndrome (IBD) through the gut-brain axis[47, 13]. These conditions are also associated with the development of a microbiota dysbiosis. (an irregularity in the microbiota within the body that can be triggered by various causes such as the effect of certain antibiotics, stress and excess of proteins and simple sugars in the diet[47], a disruption of tissue homeostasis and normal immune responses[64]).

1.2. Neurodevelopmental disorders

1.2.1 Neurodevelopmental disorders and its relationship with the gut microbiota

The combination of new technologies of microbial genomics with metabolic and immunological analyzes are revealing important synergies between the microbiota and the host. It is now possible to understand this interaction and develop new treatment strategies for major diseases such as gastrointestinal as well as neurodegenerative, which have recently show to have an important relationship with the intestinal microbiota[49].

Neurodevelopmental disorders are characterized by an alteration in the brain, behavioral and cognitive development, and sometimes as well on physical abnormalities. Some of them share abnormal behavior in socialization, communication or compulsive activity[35].

The most recognized neurodevelopmental disorder is the Autism Spectrum Disorder (ASD)[35], considered as a severe neurodevelopmental condition; it is diagnosed based on the presence and severity of stereotypic behaviors and deficit in language and social interaction[39]. The most characteristic features of the autism behavior include qualitative impairments in social interaction and communication, repetitive and stereotyped behavior patterns and a restricted repertoire of interests and activities.

Schizophrenia, considered part of the Autism Spectrum Disorder, is a chronic and severe mental disorder that affects the way a person thinks, feels and behaves[59, 61]. People with schizophrenia may seem like they have lost touch with reality. Symptoms of

schizophrenia usually begin between 16 and 30 years. In rare cases could also occur in children. The symptoms are divided into 3 categories: positive, negative and cognitive[59] (Table 1).

Table 1. Schizophrenia symptoms in patients: positive, negative and cognitive

Positive symptoms	Negative symptoms	Cognitive symptoms
Hallucinations Delusions Thought disorders Movement disorders	Flat affect Reduced feelings of pleasure in daily life Difficulty beginning and sustaining activities Reduced speaking	Poor 'executive function' Trouble focusing or paying attention Problems with working memory

While the standardized GI tract symptoms diagnosis in ASD and Schizophrenia has not yet been clearly defined, clinical and epidemiological studies have report abnormalities such as impaired GI motility and increased intestinal permeability. It has also been found that commensal bacteria affect a variety of complex behaviors including social, emotional and anxiety-related behaviors, and contribute to the development and brain function in mice and humans[8, 20, 25].

1.2.2 Prenatal infection

Prenatal exposure to infectious pathogens or inflammatory stimuli is increasingly recognized to play an important etiological role in neuropsychiatric and neurological disorders with neurodevelopmental components[60]. Significant associations between infection during pregnancy and increased disease risk in later life have been revealed for various brain disorders[32]. The British epidemiologist David Barker initially proposed this theory as the "fetal origins of adult disease" or "prenatal programing hypothesis" in the 1990`s[34]. Remote disorders, such as schizophrenia, autism, attention-deficit/hyperactivity disorder, and major depression share considerable amounts of risk

factors and brain dysfunctions, but they might lie along a continuum of genetically and environmentally induced neurodevelopmental causalities, wherein prenatal infection may be one of these factors. The nature and severity of changes at the maternal-fetal interface (Figure 1), including the placenta, amniotic fluid and fetal organism are influenced by various factors, most notably the identity or intensity of the pathogen, the gestational timing of exposure, and the genetic background of the infected host[23]. These overlapping effects are mostly characterized by increased fetal expression of inflammatory factors, such as pro-inflammatory cytokines and chemokines[27]. It is believed that abnormal expression of inflammatory factors in the fetal brain contribute to, or even mediate, abnormal brain and behavioral development following prenatal exposure to infection[7]. Indeed, as reviewed extensively elsewhere[53], acute inflammation during early fetal brain development may negatively affect ongoing neurodevelopmental processes, such as neuronal/glial cell differentiation, proliferation, migration, and survival, and thus, predispose the developing offspring to long-term brain and behavioral dysfunctions. The pathological symptoms traditionally attributed to CNS dysfunctions, neurodevelopmental psychiatric illnesses, such as autism and schizophrenia, are also associated with a number of GI dysfunctions. Such abnormalities include chronic intestinal low-grade inflammation, increased intestinal permeability “leaky gut”, allergic reactions to dietary proteins, diarrhea, gastric dysmobility, and alterations in gut microbiota[50].

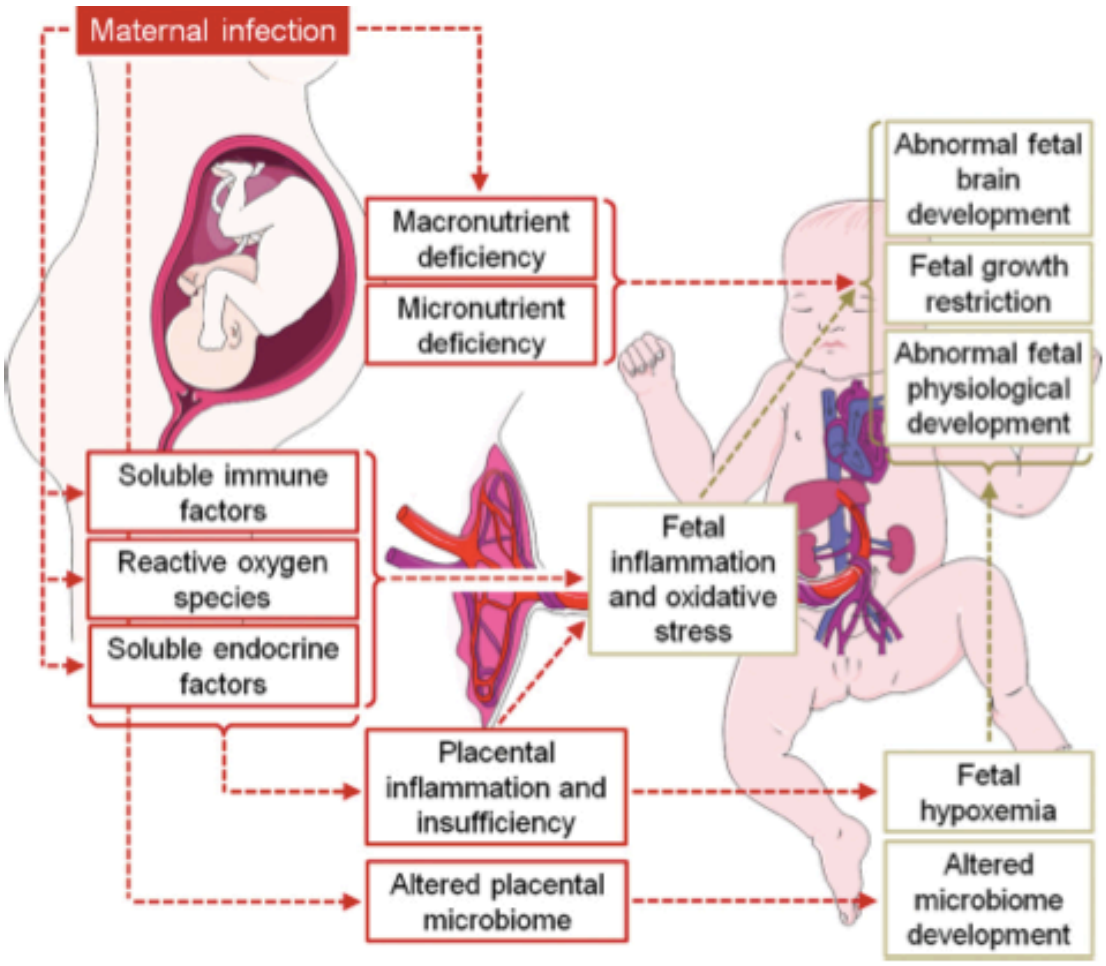


Figure 1. Possible mechanisms mediating the pathological effects of maternal infection (Adapted from [50])
Common immunological factors are key mediating factors changing developmental trajectories in the offspring. Inflammatory cytokines are typically induced during the acute phase response to infection and may represent a major developmental stressor for the organism. An increase in fetal cytokine levels may be caused by transplacental transfer of maternally produced cytokines, by placental production of cytokines, or by increased fetal cytokine synthesis. In addition, there is a strongly association with numerous other pathophysiological effects, including oxidative stress, referred to as an imbalance between the production and elimination of reactive oxygen species (ROS), some of which are highly cytotoxic and promote tissue injury. Upon activation, innate immune cells secrete ROS and reactive nitrogen species (RNS) as a central part of killing invading pathogens. Production of ROS and RNS is, thus, an important downstream mechanism of inflammation-mediated immune responses. Activation of the innate immune system (in response to infection) also changes the maternal and fetal availability of several micronutrients, including iron and zinc, both of which are highly important for the normal development of peripheral and central organs. In the case of iron, it is well established that infection leads to a temporary depletion of iron in the infected host. This process is mediated to a great extent by the pro-inflammatory cytokines IL-1β and IL-6 and serves to reduce the availability of this essential nutrient to the invading pathogens as part of the host's inherent defense system. As part of the acute-phase response to infection, pro-inflammatory cytokines also trigger the induction of the zinc-binding protein metallothionein. During the course of pregnancy, this process leads to maternal and fetal zinc deficiency, which has further been associated with teratogenicity and abnormal developmental processes in utero. In addition may also impair the fetal supply for macronutrients. Indeed, it is well established that peripheral cytokine elevation in response to infection induces a set of behavioral and physiological changes collectively referred to as sickness behavior. Sickness behavior typically includes fever, malaise, and reduced exploratory, and social investigation, as well as decreased food and water intake, usually accompanied by weight loss. It should also be noted that at least part of the changes to the microbiome that emerge following prenatal infection might have an early prenatal origin. The conventional view is that microbial colonization begins at birth when the neonate is first exposed

to the microbiome of the mother and the surrounding environment, implying furthermore that the fetal environment is sterile and, therefore, lacks a microbiome before birth. Recently findings show that the human placenta is not sterile but, in fact, is colonized with nonpathogenic commensal microbiota. Perhaps even more important are the findings suggesting that the microbial composition of the human placenta can be modified by maternal infection during pregnancy, even if the infectious process takes place during the time of conception or in early gestation. Modifications of the placental microbiome, be it as a result of maternal infection or by other environmental factors, can critically shape the development of the offspring's microbiome and, thus, predispose the developing organism to dysbiosis and other microbiome-associated abnormalities.

1.2.2.1 Prenatal infection animal models

With the knowledge that the alteration of neuroimmune mechanisms may play a role in the development of schizophrenia and other related psychotic illnesses, a number of mouse models have been established to explore the consequences of prenatal immune activation on the brain and the behavioral development. Existing models are normally based on a gestational maternal exposure to a specific infectious disease agent or an inflammatory marker as, for instance: the human influenza virus, the mimetic viral mimetic polyriboinosinic-polyribocytidilic acid [poly(I:C)], the lipopolysaccharide bacterial endotoxin or selected inflammatory cytokines[52]. Several of these mouse models show that the maternal exposure to infectious agents or immune system activation lead to post-acute robust immunological changes in the maternal-fetal interface, including placenta, amniotic fluid and the fetus organism itself.

1.2.2.1.1The poly(I:C) model. One of the most robust and widely used method that uses immune activation agents, which evoke associated cytokines to the mother immune response without using live viral or bacterial pathogens, is the maternal administration of the poly(I:C)[51]. This prenatal poly(I:C) model has had a significant impact on researchers who focus on the basis of neurological and neuroimmune development of complex brain disorders such as schizophrenia[52]. In poly(I:C) model, pregnant mouse

dams are exposed to the immunological manipulation at a specific gestational stage, and the brain and behavioral consequences of the prenatal immunological manipulation are then compared in the resulting offspring relative to offspring born to vehicle-treated control mothers. Poly(I:C) is a commercially available synthetic analog of double-stranded RNA[52]. Double-stranded RNA is generated during viral infection as a replication intermediate for single-stranded RNA or as a by-product of symmetrical transcription in DNA viruses[26]. It is recognized as foreign molecule by the mammalian immune system primarily through the transmembrane protein toll-like receptor 3 (TLR3)[5]. Administration of poly(I:C) can therefore efficiently mimic the acute phase response to viral infection and leads to significant inflammatory processes in the fetus when given systemically to pregnant mouse dams[52]. An interesting feature of this prenatal poly(I:C) mouse model, is that the spectrum of behavioral, cognitive, and pharmacological disorders emerge only after the offspring has reached late adolescence or early adulthood[50, 63]. This ripening delay is indicative of a progression of pathological symptoms from puberty to adulthood, which is consistent with post-pubertal apparition of psychotic behavior in schizophrenia and other ASD related disorders[52].

1.2.2.1.2 Behavioral phenotypes in first- and second-generation offspring of immune-challenged mothers

A previous research[17] evaluated the transgenerational transmission and modification of pathological traits induced by prenatal immune activation, using an established mouse model of prenatal immune activation by the viral mimetic poly(I:C), behavioral phenotypes emerging in F_1 and F_2 offspring of poly(I:C)-exposed mothers relative to corresponding control offspring were compared. For each generation, behavioral testing

started when the offspring reached postnatal day (PND) 70 and included tests assessing social interaction, cued Pavlovian fear conditioning, prepulse inhibition (PPI) of the acoustic startle reflex, and behavioral despair in the forced swim test. They found that reduced sociability (Figure 2) and increased cued fear expression (Figure 3) are similarly present in the first (F_1) and second (F_2) generation offspring of immune-challenged ancestors, that the sensorimotor gating impairments (Figure 4) are confined to the direct descendants of infected mothers, whereas increased behavioral despair (Figure 5) emerges as a novel phenotype in the F_2 generation. These transgenerational effects are mediated via the paternal lineage (Figure 6), demonstrating transgenerational non-genetic inheritance of pathological traits following in-utero immune activation.

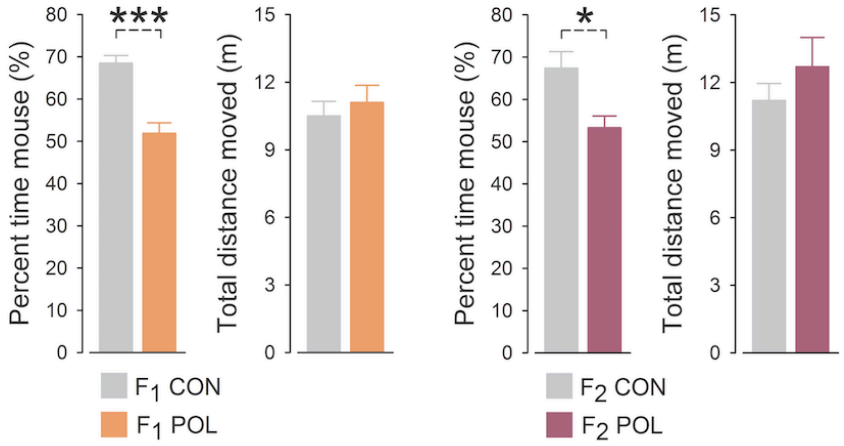


Figure 2. F_1 poly(I:C)-exposed offspring displayed impaired sociability in a social interaction test, in which they were allowed to concomitantly explore an inanimate dummy object and an unfamiliar live mouse. Whereas F_1 control offspring showed a strong preference for the live mouse versus the inanimate dummy object, F_1 poly(I:C) offspring did not display such a preference. Moreover, F_1 control and poly(I:C) offspring explored the dummy object to a similar extent, whereas the exploration times for the live mouse markedly differed between the two groups. F_2 offspring of poly(I:C)-exposed ancestors showed a similar deficit in sociability without concomitant changes in general locomotor activity or dummy object exploration.

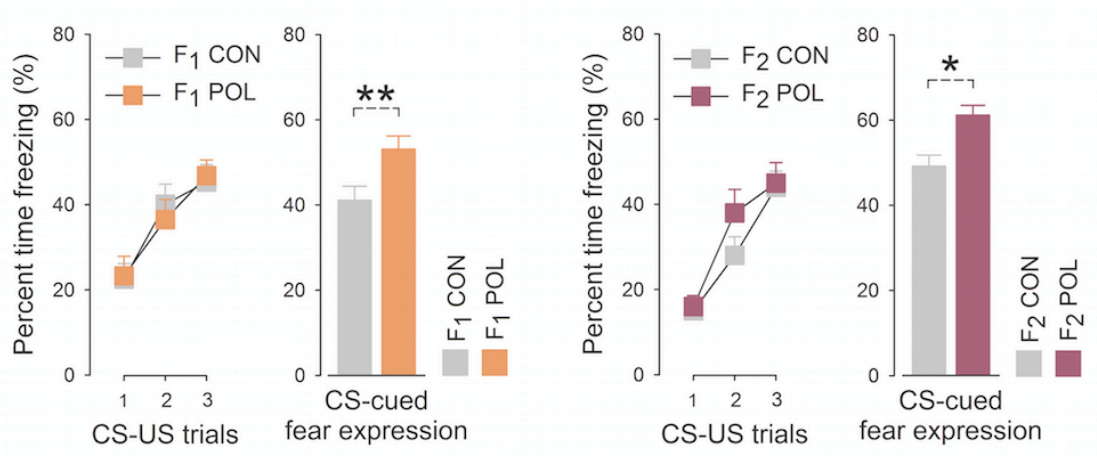


Figure 3. F_1 poly(I:C) offspring displayed increased fear expression in a cued Pavlovian fear conditioning test. Whereas they did not differ from F_1 controls during the initial acquisition of the conditioned fear response to successive $CS_{(tone)}$-$US_{(foot\ shock)}$ presentations, F_1 poly(I:C) offspring showed increased conditioned fear when the CS was no longer followed by the US. Similar fear-related abnormalities were also present F_2 offspring of poly(I:C)-exposed ancestors: Whilst the acquisition of the fear response during initial tone-shock conditioning was not different between F_2 control and F_2 poly(I:C) offspring, the latter displayed increased cued fear expression in the subsequent test phase.

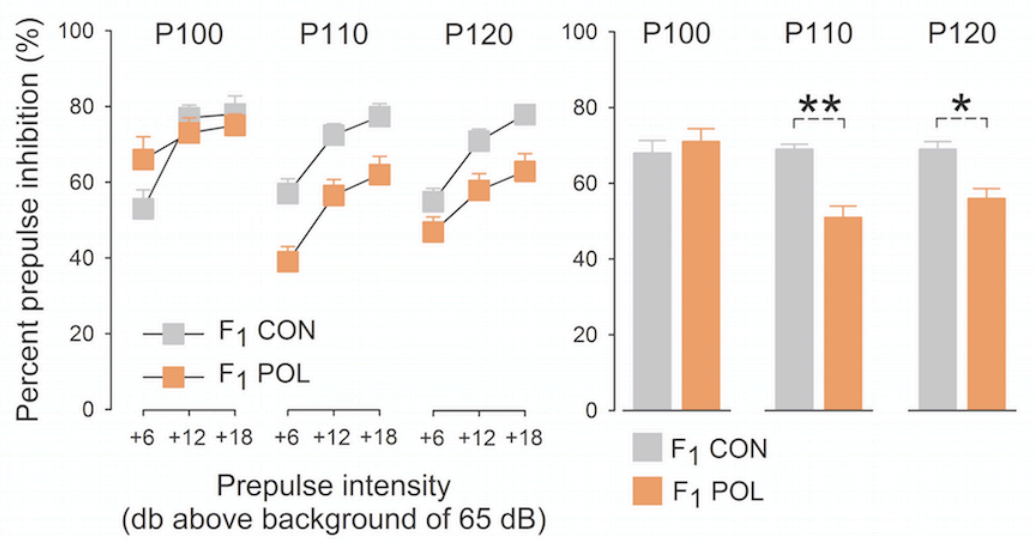


Figure 4. F_1 poly(I:C) offspring displayed a robust reduction in PPI scores when 110 and 120 dB stimuli served as pulses. These effects were not associated with changes in the responses to prepulse-alone or pulse-alone trials (data not shown), suggesting that prepulse detection and startle reactivity per se were not affected by prenatal immune activation. F_2 offspring derived from poly(I:C)-exposed or control ancestors showed comparable PPI and responses to prepulse- and pulse-alone trials (data not shown). Hence, despite the robust PPI deficits in the F1 generation of immune-challenged mothers, the F_2 generation did not inherit the sensorimotor gating deficit.

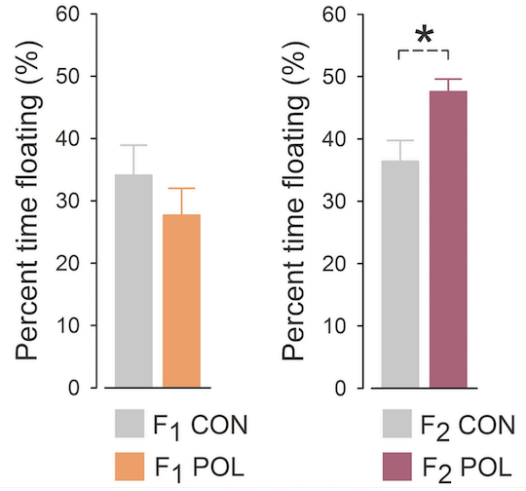


Figure 5. To examine affective behaviors in F_1- and F_2-generation offspring of immune-challenged mothers, the forced swim test was used. F_1 poly(I:C) offspring did not differ from F_1 control offspring with regards to the time spent floating. Despite the absence of behavioral despair in the F_1 generation, however, F_2 offspring of poly(I:C)-exposed ancestors spent significantly more time floating than F_2 control offspring. Hence, F_2 but not F_1 offspring derived from immune-challenged mothers develop signs of behavioral despair.

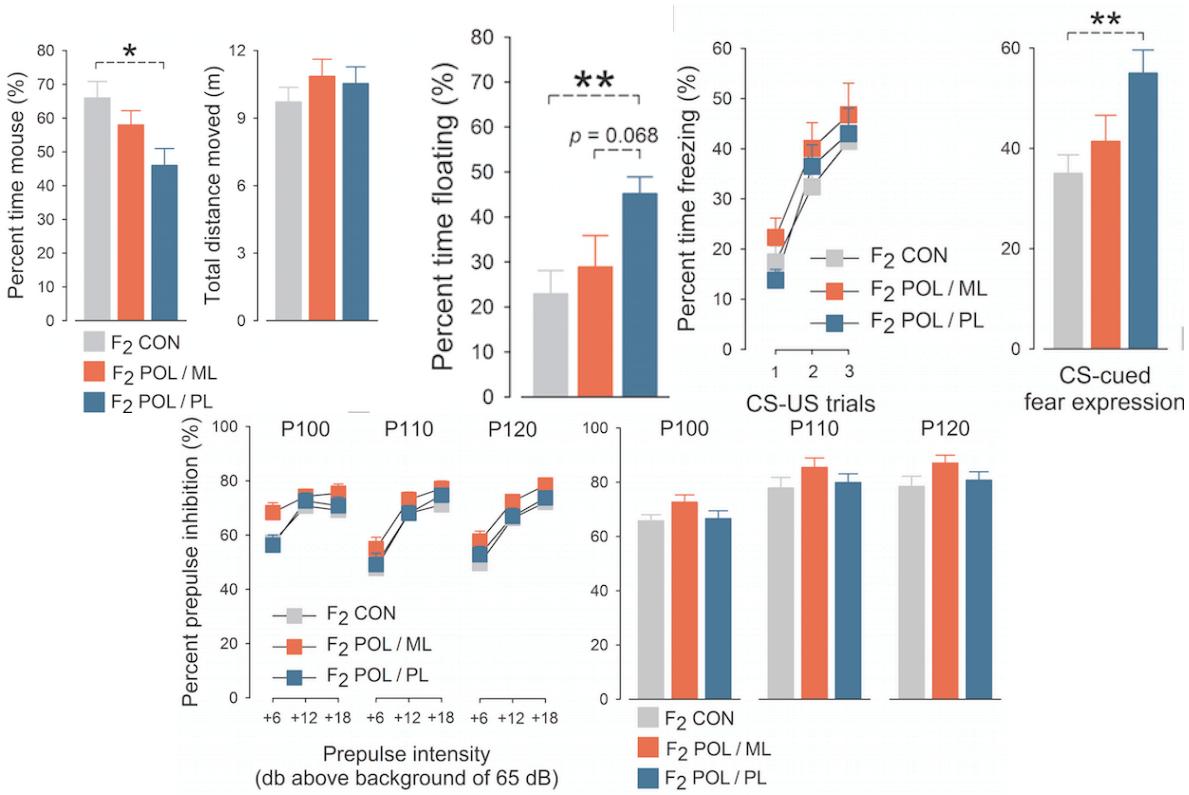


Figure 6. Paternal lineage (PL)-derived F_2 poly(I:C) offspring showed deficits in social interaction, increased fear expression and behavioral despair, but not sensorimotor gating. The former behavioral abnormalities were not associated with changes in basal locomotor activity or altered acquisition of the fear response during initial tone-shock conditioning. Interestingly, F_2 poly(I:C) offspring did not display overt behavioral impairments when they were derived from the maternal lineage (ML) ancestor lineage. Demonstrating that the transgenerational transmission of behavioral deficits following prenatal immune activation is mediated via the PL but not ML.

1.3. Study of microbial communities

1.3.1 Taxonomy

The taxonomy is a division of the systematic field in biology; related with the classification of organisms according specialization[31]. It provides the methods, principles and rules for the classification of living organisms into taxa (categories)[1].

The categories consist of levels within groups in which the largest group covers the small one. Grouping organisms is based on similarities and differences, both natural (structural) and phylogenetic (family relationships or affinities with other missing organisms). The taxonomy provides direct and inferential information on the body structure and evolutionary history of organisms respectively. The most general taxonomic level is the Kingdom, which is divided then in other categories such as *Phylum*, *Class*, *Order*, *Family*, *Genus*, and *Specie*[31].

1.3.2 Metagenomics.

Phylogenetic relationships (evolutionary) among microorganisms using “molecular characters” can be determined. For establishing these relationships, a series of methods based on the comparison of nucleic acid sequence, particularly the sequence of ribosomal RNA (rRNA, structural RNA ribosome, involved in RNA translation) are used[31]. One of the most important recent discoveries in biology is that the changes in the nucleotide sequence of ribosomal RNA (determined by mutations in the DNA encoding ribosomal RNA) can be used as a measure to establish evolutionary relationships between organisms.

Metagenomics is a new field in which it seeks to obtain genome sequences of different microorganisms, such as bacteria, which makes up a community, globally extracting and analyzing their DNA. The ability to directly sequence the genomes of microbes without cultivate opens new possibilities involving a change in microbiology. This is a scientific revolution because of its high performance and low cost that allows the access to the genome without seeing or cultivating microorganisms[2].

1.3.3 Bacterial identification by sequencing of ribosomal genes (16S rRNA)

Figure 7. 16SrRNA structure, conserved regions and hipervariables regions (V1-V9)[46]

To perform genome sequencing of microorganisms by analyzing their DNA, ribosomal RNA (rRNA) genes are used[24]. The rRNA gene is the most conserved DNA in the cells[46]. Portions of the rRNA sequence of distantly related organisms are remarkably similar, meaning that sequences of distantly related organisms can be aligned accurately, so that differences become easily measured. Therefore, rRNA encoding

genes become widely used to determine taxonomy and phylogeny, and also for estimating rates of divergence between bacterial species[30].

The 16S rRNA is the most widely used macromolecule in studies of bacterial phylogeny and taxonomy. Its application as a molecular clock was proposed by Carl Woese in the early 1970s, is the "target" gene most commonly used in studies of bacterial diversity, known as a universal marker relatively unaffected by environmental pressures over time. 16S rRNA contains about 1,500 basepairs[46].

16S rRNA contains two types of regions, hypervariable regions, where the sequences have been distanced by the evolutionary time designated as V1-V9 and strongly conserved regions that often flanked hypervariable regions (Figure 7)[46]. Specific primers are designed to bind to the conserved regions and amplify the variable regions.

The sequence analysis of the16S rRNA of various phylogenetic groups revealed the presence of one or more characteristic sequences, which are termed “signature oligonucleotides”. Therefore, signature oligonucleotides can be used to locate each bacterium within their own group[16].

The DNA sequence of the gene 16S rRNA has been determined for an extremely large number of species[24, 30]. Sequences of tens of thousands of clinical and environmental isolates are available through various public databases, with free internet access, such as GenBank NCBI (National Center for Biotechnology Information), EMBL (European Molecular Biology Laboratory), Greengenes, RDP (Ribosomal Database Project), RIDOM (Ribosomal Differentiation of Medical Microorganisms), and other private

databases, as MicroSeq (Applied Biosystems), and SmartGene IDNS (Integrated Database Network System).

It is important to keep in mind, that is the comparison of the complete genomes, and no the comparison of 16S rRNA, which provides an accurate indication of the evolutionary relationships. In its absence, the bacterial species are defined in taxonomy, as the set of strains that share a similarity of 70% or more. Experiments show that strains with this level of relatedness typically have a 97% identity or more between their 16S rRNA genes. Thus the strains with less than 97% identity in the16S rRNA sequences are unlikely to be related to species[12]. Today, the accepted species classification can only be achieved by the recognition of genomic distances and limits between the closest classified taxons (DNA–DNA similarity), and of those phenotypic traits that are exclusive and serve as diagnostic of the taxon (phenotypic property)[70].

The molecular identification method of bacteria by sequencing the 16S rRNA includes three successive stages: 1) gene amplification from the appropriate sample, 2) determining the nucleotide sequence of the amplicon, and 3) sequence analysis.

1.3.4 Next Generation Sequencing

DNA sequencing is a set of methods and biochemical techniques aimed at determining the order of nucleotides (adenine; A, cytosine; C, guanine; G and thymine; T) in a DNA oligonucleotide. Currently one of the most used techniques is the massive sequencing or "Next Generation Sequencing" (NGS) which allows for millions of sequences in the same process.

The continuous development of NGS, has led to a rapid increase in the amount of genomic data generated for processing and analyzing. Unlike traditional sequencing systems, these massive sequencing platforms, are capable of generating parallel and massively, millions of DNA fragments in a single sequencing process in record time and cost shrinking. Because of its high performance, this type of platform is ideal for numerous studies on a large scale impossible to address with any other existing technology, due to the enormous cost that this would entail.

1.3.5 Next generation sequencers

Different platforms carry out the NGS; the most common are *Ion Torrent PGM* (Thermo Fisher Scientific Inc)[19], *MiSeq* (Illumina Inc), and *454 Life Sciences* (Hoffmann-La Roche). The following explains the biochemical and physical principles of the two most commonly used NGS platforms:

1.3.5.1 Ion Torrent PGM (IT)

Ion Torrent PGM (Personal Genome Machine) directly translates chemical encoded information (A, T, G, C) into digital information (0,1), in a semiconductor chip, containing millions of wells that capture chemical DNA sequencing information and convert it into digital information[19].

The sequencing process begins when a DNA sample is cut into millions of fragments, which will bind to its complementary sequence found in each of the microspheres that will pass through wells, copies of each of these fragments were held covering the entire microsphere. These process covers millions of microspheres with millions of different

fragments. The microspheres will flow through the chip and will deposit in each of the wells (by probabilistic chance). Thereafter, the chip is immersed by 1 of the 4-nucleotide dNTP solutions (Deoxynucleotide Solution Mix), which contains equimolar nucleotides concentrations of: dATP, dCTP, dGTP and dTTP. When a nucleotide is incorporated into a DNA strand and is immersed in one of the nucleotide solutions, a hydrogen ion is released. The released hydrogen produces pH changes in each one of the wells, creating a voltage difference. This voltage change is registered, which indicates that a nucleotide has been incorporated. The process is repeated every 15 s with a different nucleotide solution and is carried out simultaneously in millions of wells (Figure 8)[19] .

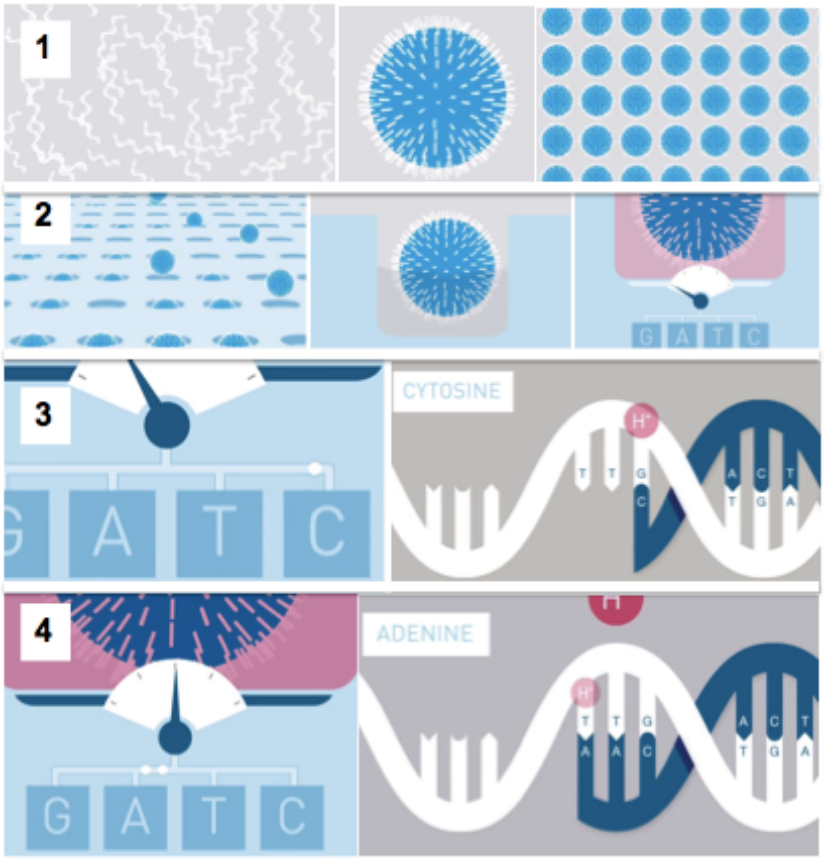


Figure 8. Ion Torrent PGM (IM) sequencing process (adapted from[19]). Four processes from left to right:

1 DNA sample cut into million fragments bind to the spheres, copied and coating the entire area, the process is carried out in millions of spheres. **2** Spheres flow through the chip and deposited in each of the wells, the chip is immersed in one of the four equimolar solutions and when a nucleotide is incorporated into a DNA strand a hydrogen ion is released causing pH changes generating a voltage difference. **3** Example: A polymerase incorporates a cytosine nucleotide in the DNA strand, having a complementary nucleotide (guanine) in sequence, a release of a hydrogen, a pH change and a voltage difference will occur **4** Example 2: There are two identical bases together A-A (adenine), so two nucleotides are incorporated, there will be two hydrogen ions released one by each of the joints, the voltage will double and 2 continuous bases registered.

1.3.5.2 Illumina MiSeq (IM)

Illumina MiSeq NGS, uses clonal amplification and chemical synthesis sequencing to allow a rapid and accurate sequencing. The process (Figure 9) simultaneously identifies DNA bases and their incorporation into a nucleic acid strand. Each base emits a single fluorescent signal, as it is added to the growing chain, using this to determine the order of the DNA sequence. The IM sequencing method is similar to Sanger sequencing, but it uses modified dNTPs containing a terminator which blocks further polymerization, so a polymerase enzyme to each growing DNA copy strand can add only a single base.

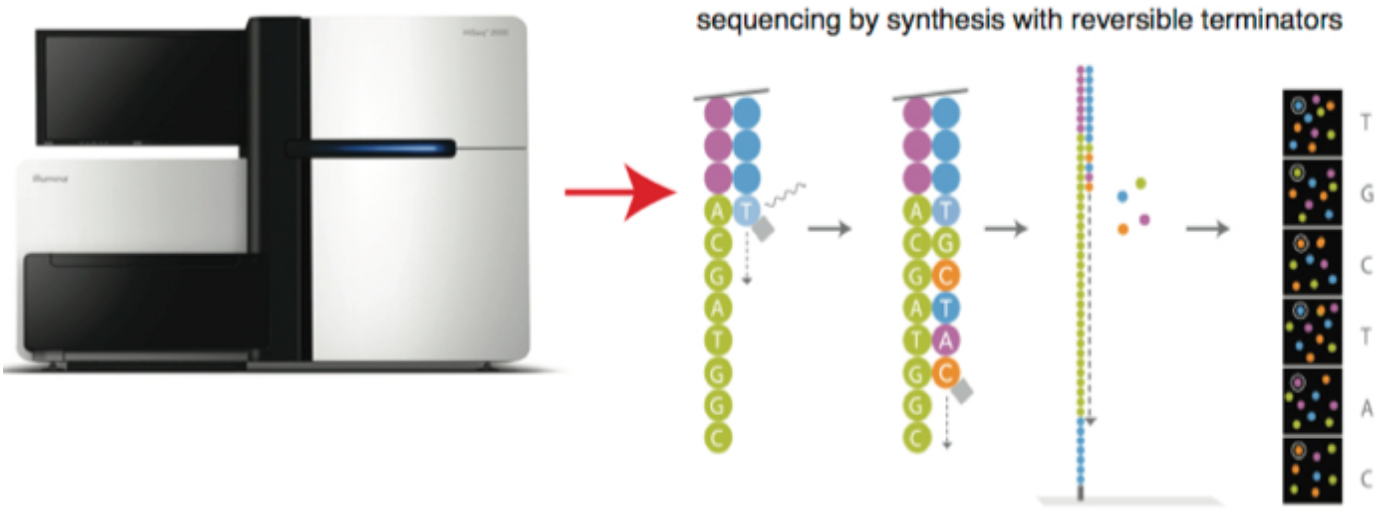


Figure 9. Illumina (IM) sequencing process[29]
From left to right. During sequencing, we have the primer and the fluorophores, a laser comes and excites the molecule, the fluorophore will be released emitting a color spectrum which is specific for each nucleotide, once the issue occurs, the computer has a high definition camera, which takes a picture to

each of the spots, as the fluorophore is gone polymerization can occurs and the next nucleotide arrives, repeating the process.

The sequencing reaction is conducted simultaneously on a very large number (many millions) of different template molecules spread out on a solid surface. The terminator also contains a fluorescent label, which can be detected by a camera. Only a single fluorescent color is used, so each of the four bases must be added in a separate cycle of DNA synthesis and imaging. Since single bases are added to all templates in a uniform fashion, the sequencing process produces a set of DNA sequence reads of uniform length[29].

General differences among NGS platforms exist, including relative turnaround times, per-base sequencing costs, read lengths, and several accuracies as shown in Table 2.

Table 2. Technical specifications of Next Generation Sequencing platforms (IM-Ion Torrent PGM and IM-Illumna MiSeq). Obtained from[3] .All cost calculations are in dollars

	IM-Ion Torrent PGM	**IM-Illumina MiSeq**
Principle of addition of nucleotides during DNA synthesis	Prepares templates by using emulsion PCR	DNA fragments are prepared by isothermic "bridge PCR".
Error rate	The error rate averages of 1.5 and 1.4 errors per 100 bases for read sequences from the forward and reverse directions, respectively	The error rate average of 0.9 errors per 100 bases for read sequences from the forward and reverse directions, respectively
Sequence yield per run	20-50 Mb on 314 chip, 100-200 Mb on 316 chip, 1Gb on 318 chip	1.5-2Gb
Run Time	2 hours	27 hours
Reported Accuracy	Mostly Q20	Mostly > Q30
Read length	~200 bases	up to 150 bases
Paired reads	Yes	Yes
Insert size	up to 250 bases	up to 700 bases
Typical DNA requirements	100-1000 ng	50-1000 ng
Instrument Cost	$80 KUSD	$128 KUSD
Sequencing cost per Gb*	$1,000 USD (318 chip)	$502 USD

1.3.6 Biodiversity

Biodiversity is defined as "the variability among living organisms from all sources; this includes diversity within species, between species and of ecosystems[41]". To understand the changes in biodiversity, separation of components or indexes alpha, beta and gamma can be very useful. Alpha diversity is the wealth of species in a particular community which is consider as homogeneous, beta diversity is the degree of change or replacement in species composition between different communities, and gamma diversity is species richness of all communities that are part of an ecosystem, resulting both alpha and beta diversities[56]. It is important to mention that this work focuses on structure and diversity of bacterial communities.

1.3.6.1 Alpha diversity metrics. For full parameters of the diversity of species in a habitat, it is advisable to quantify the number of species and their representativeness. There are indexes that summarize a lot of information into a single value and allow us to make quick comparisons and subject to statistical verification between the diversity of different habitats and the diversity of the same habitat over the time[41, 56].

a) *Chao1 diversity index.* It is an estimate of the number of species from a community based on the number of rare species in the sample Chao 1 is a nonparametric model[42].

$Chao1 = S + \frac{a2}{2b}$ (S is the number of species in a sample; 'a' is the number of represented species only by a single individual in that sample and 'b' is the number of species represented by exactly two individuals in the sample).

b) *Shannon diversity index*. It is a commonly used index to characterize the diversity of species in a community, just as the Simpson index, Shannon index represents both the abundance and uniformity of the present species[42].

$H = -\sum_{i=1}^{\infty} p_i \, lnp_i$ The relative proportion of species *(i)* is calculated with the total number of species *(pi)*

c) *Simpson diversity index.* A community dominated by one or two species is considered less diverse than one where different and various species have a similar abundance. Simpson diversity index is a metric of diversity that takes into account the number of present species and the relative abundance of each species. So if richness and uniformity of species increase, the diversity increases[42].

$D = 1 - (\frac{\sum n\,(n-1)}{N(N-1)})$ (n=total number of organisms of a particular species, and N=total number of organisms of all species).

d) *Simpson reciprocal diversity index.* This is an index that increases with diversity rather than decrease. As its name says it will calculate the inverse of Simpson index[42].

$\Delta = \frac{1}{D}$ Taking D as the probability of an intra specified meeting, which will increase when the community is less equitable.

invD=1-D

e) *Rarefaction curves.* Another way of exemplifying alpha diversity is through rarefaction curves. Rarefaction is a technique for assessing species richness from sampling results. Allows calculation of species richness for a given individual samples, based on the construction of rarefaction curves. These curves are a graphical representation of the number of species as a function of the number of

samples. The steep slope indicates the fraction of the diversity of species, which remains to be discovered, if the curve approaches asymptotically a maximum, it means that a reasonable number of individual samples have been taken[41].

1.3.6.2 Beta diversity metrics. The beta diversity or diversity between habitats is the degree of replacement of species or biotic change through environmental gradients, measuring beta diversity is based on ratios or differences[41]. These ratios can be evaluated based on indexes or coefficients of similarity, dissimilarity or distance between samples from qualitative (presence - absence of species) or quantitative data (proportional abundance of each species measured as number of individuals, biomass, density, coverage, etc.) as well as Heat maps, dendrograms, PLS-DA (Partial Least Squares Discriminant Analysis) or Principal Component Analysis PCA and Principal Coordinates Analysis PCoA graphs[42].

1.7 Massive data processing

1.7.1 Bioinformatic analysis

Bioinformatics is an emerging and relatively new discipline that integrates disciplines as biology, computer science, statistics and mathematics[19]. Bioinformatics comes as a response to the exponential increase in the volume of data generated by the scientific community over the last decade caused by the development of new high-performance technologies such as microarrays and next generation sequencing. The difficulty of managing a growing volume of data makes it necessary to develop new bioinformatic

solutions that facilitate the transformation of raw data produced in biological processes, so that we can advance in the understanding of the molecular processes involved. In the case of the next generation sequencing, there are many methods and bioinformatics tools for analyzing this obtained data, the preference of each individual for programs and platforms through graphic interfaces or terminal and also the power or characteristics of the available computer equipment or server.

Some of these tools to perform the data analysis for the sequenced DNA microbiome involve using virtual machines as *Bio Linux* or *Clovr* containing a set of programs used to carry out the entire sequence analysis, open source web platforms as *Galaxy* where all information is kept in the "cloud" (http://galaxy-qld.genome.edu.au/galaxy), or open source bioinformatic pipelines and algorithms for raw data as *Qiime* (http://qiime.org), *Mothur* ((http://www.mothur.org), *Uparse, Uchime, Usearch, Ublast, Uclust* among others. Similarly, exist independent programs that allow carrying out some part of data processing as *Trimommatic* and *FastQC* (Babraham Institute, Cambridge UK). It is important to note that the person carrying out this type of analysis must have knowledge of commands through (Linux) terminal, since most tools require it.

1.7.2 Bioinformatic tools for sample analysis

QIIME ™ (Quantitative Insights Into Microbial Ecology; www.quime.org)[43]. *QIIME* is an open-source bioinformatics pipeline for performing microbiome analysis from raw DNA sequencing data. It has been used to analyze and interpret data from nucleic acid sequences of fungi, bacteria, viruses and archaea. *QIIME* is designed to take users from raw sequencing data generated on the IM, IT or other platforms through

publication quality graphics and statistics. This includes demultiplexing and quality filtering, OTU picking, taxonomic assignment, and phylogenetic reconstruction, and diversity analyzes and visualizations. *QIIME* has been applied to studies based on billions of sequences from tens of thousands of samples[43]. Using *QIIME* to analyze data from microbial communities consists of typing a series of commands into a terminal window, and then viewing the graphical and textual output. Some fairly basic familiarity with a Linux-style command-line interface is useful[44].

Trimmomatic[18] . *Trimmomatic* is a bioinformatics tool that performs a variety of useful tasks of trimming and quality analysis of the sequences. The selection of trimming steps and their associated parameters are supplied on the command line. The trimming steps are:

- ILLUMINACLIP: Cut adapter and other Illumina-specific sequences from the read.
- SLIDINGWINDOW: Perform a sliding window trimming.
- LEADING: Cut bases off the start of a read, if below a threshold quality
- TRAILING: Cut bases off the end of a read, if below a threshold quality
- CROP: Cut the read to a specified length
- MINLEN: Drop the read if it is below a specified length
- TOPHRED33: Convert quality scores to Phred-33

FASTQC[28]. *FastQC* is a quality control tool for high throughput sequence data. *FastQC* aims to provide a simple way to do some quality control checks on raw sequence data coming from high throughput sequencing pipelines. It provides a modular set of analyzes, which can be use to give a quick impression of whether the data has any

problems of which should be aware before doing any further analysis. The main functions of *FastQC* are:[28]

- Import of data from BAM, SAM or FastQ files (any variant)
- Providing a quick overview to tell you in which areas there may be problems
- Summary graphs and tables to quickly assess your data
- Export of results to an HTML based permanent report

USEARCH and UBLAST[36]. *UBLAST* and *USEARCH* are new algorithms enabling sensitive local and global search of large sequence databases at exceptionally high speeds. They are often orders of magnitude faster than *BLAST* in practical applications, though sensitivity to distant protein relationships is lower. *UCLUST* is a new clustering method that exploits *USEARCH* to assign sequences to clusters. *UCLUST* offers several advantages over the widely used program *CD-HIT*, including higher speed, lower memory use, improved sensitivity, clustering at lower identities and classification of much larger datasets.[36]

UPARSE[37]. *UPARSE*, is a pipeline which reports operational taxonomic unit (OTU) sequences with 1% incorrect bases in artificial microbial community tests, compared with >3% incorrect bases commonly reported by other methods. The improved accuracy results in far fewer OTUs, consistently closer to the expected number of species in a community. It works by quality-filtering reads, trimming them to a fixed length, optionally discarding singleton reads and then clustering the remaining reads, performs chimera filtering and OTU clustering simultaneously. It does not require technology or gene-specific parameters, algorithms or data, which makes it highly robust and suggests that

could be successfully applied to a wide range of marker genes and sequencing technologies[37].

UCHIME[62]. Chimeric DNA sequences often form during polymerase chain reaction amplification, especially when sequencing single regions to assess diversity or compare populations. Undetected chimeras may be misinterpreted as novel species, causing inflated estimates of diversity and spurious inferences of differences between populations. Detection and removal of chimeras is therefore of critical importance in such experiments. *UCHIME* is a new program that detects chimeric sequences with two or more segments. It either uses a database of chimera-free sequences or detects chimeras *de novo* by exploiting abundance data. *UCHIME* has better sensitivity than ChimeraSlayer (previously the most sensitive database method), especially with short, noisy sequences. UCHIME is >1000× faster than ChimeraSlayer[62].

Databases. It is important to mention that to carry out the processing of massive sequencing data, one of the most important steps is the OTU picking, which needs the use of a database as reference. 16S rRNA gene sequence and hyper variable regions have been determined for a number of organisms, and are available in various free access databases such as: *Greengene*s (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi), *SILVA* (http://www.arb-silva.de), *RDP* (Ribosomal Database Project) (http://rdp.cme.msu.edu/) among others, and choosing one or another depends on the researcher and the project needs or preferences.

2. Justification

About 1 in 6 (16%) children in the world had a developmental psychoneurological disability (2006-2008), such as speech and language impairments to serious ones, as intellectual disabilities, cerebral palsy, and autism[11]. The prevalence of the Autism Spectrum Disorder (ASD) is being increasingly in the last years, about 1 in 68 (1.5%) children have been identified with ASD according to estimates from Autism and Developmental Disabilities Monitoring Network[6]. The total costs per year for children with ASD in the US were estimated to be between $11.5-$60.9 billion USD. Representing a variety of direct and in-direct costs, from medical care to special education to lost parental productivity. On average, medical expenditures for children with ASD were 4.1–6.2 times greater than for those without ASD[14].

Schizophrenia is a mental disorder considered as part of the Autism Spectrum Disorder. Although there is no cure (as of 2007) for schizophrenia, the treatment success rate with antipsychotic medications and psychosocial therapies can be high. After 30 years, of the people diagnosed with schizophrenia[22]:

- 25% Completely Recover
- 35% Much Improved, relatively independent
- 15% Improved, but require extensive support network
- 10% Hospitalized, unimproved
- 15% Dead (Mostly Suicide)

Today the leading theory of why people develops Schizophrenia is that it is a result of a genetic predisposition combined with an environmental exposures and stress during

pregnancy or childhood that contribute to, or trigger, the disorder[14]. The prevalence rate for schizophrenia was approximately 1.1% of the population over the age of 18 in 2013[22]. Schizophrenia is a devastating disorder for most people who are afflicted, and very costly for families and society. The overall U.S. 2002 cost of schizophrenia was estimated to be $62.7 USD billion, with $22.7 USD billion excess direct health care cost ($7.0 billion USD outpatient, $5.0 billion USD drugs, $2.8 billion USD inpatient, $8.0 billion USD long-term care)[15].

These neurodevelopmental diseases are disorders that are present in our society and its incidence is increasing every day, therefore, is important to continue investigating and trying to find new solutions using latest technologies. NGS is arguably one of the most significant technological advances in the biological sciences over the last 30 years[4]. An increasingly diverse range of biological problems is harnessing the power NGS technologies. Nowadays, is expected to help find the elusive, causative genetic defects associated with neurodevelopmental disorders, such as the relationship that exists between the brain and the gut microbiota. In comparison with traditional sequencing, the use of NGS is regarded as ideal to discover genetic mutations and gene expression variations causative of neurodevelopmental disorders because of the amount and diversity of genetic variants these technologies can reveal. Biomedical research can provide a great amount of new information, which, at times, is unrelated to the issue that first prompted the study. This series highlights the breadth of next–generation sequencing applications and the importance of the insights that are being gained through these methods[4].

3. Hypothesis

If the gut microbiota is a relevant factor on the etiology of schizophrenia, a microbial dysbiosis will be observed in the mouse model of prenatal infection, which might work as a microbial vector that might be also horizontally transmitted across generations.

4. Main objective

To demonstrate the influence of the gut microbiota in neurodevelopmental disorders by applying bioinformatics algorithms for processing data from the next generation microbial genome sequencing. For which the characterization of gut microbiota based in an established mouse model of prenatal immune activation by the viral mimetic poly(I:C), was raised.

4.1 Specific objectives

I. To compere the gut microbiota composition in relative abundance and diversity of the F_1 generation of immune challenged mice by the prenatal treatment with poly(I:C) and the vehicle control mice.

II. To compare the results of two different NGS platforms (Illumina Miseq vs. Ion Torrent PGM).

III. To assess whether there is a transmission of bacterial communities that are causing dysbiosis from the F_1generation to a F_2 generation.

IV. To evaluate whether there is a preponderant transmission linage (maternal vs. paternal) that is relevant for the transmission of bacterial communities from the generation $F_1$to a generation F_2.

5. Materials and methods

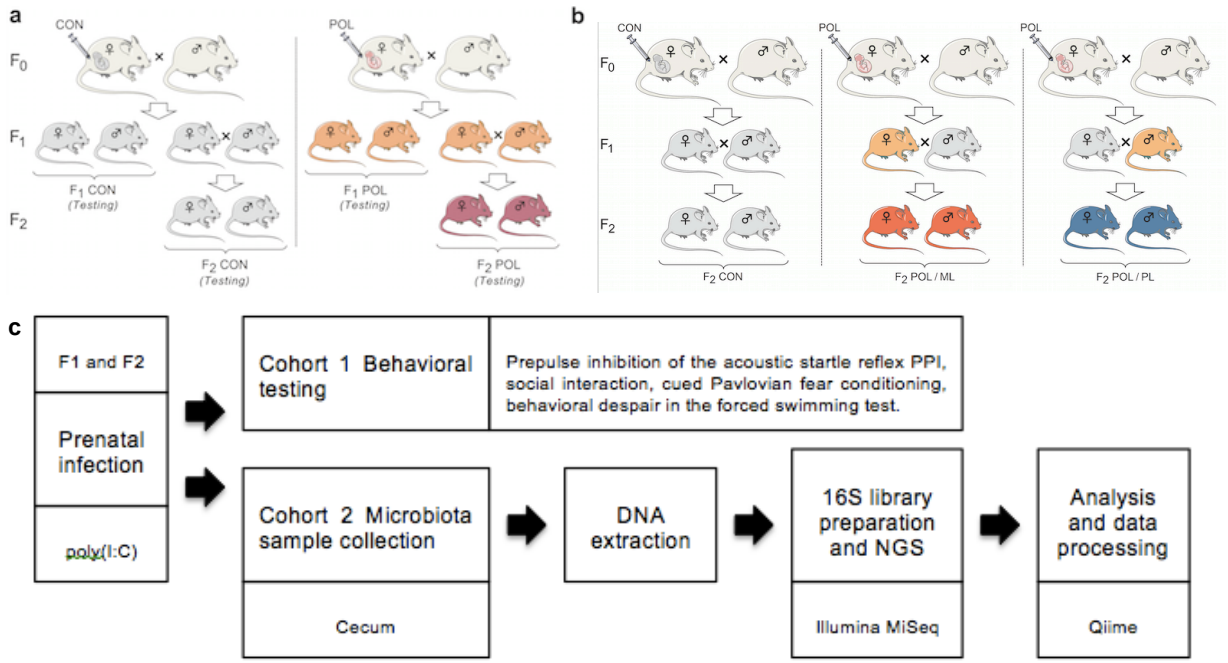


Figure 10. (Adapted from [17]) a) F_1 males born to poly(I:C)-exposed mothers were mated with F_1 females born to poly(I:C)-exposed mothers (*N* = 6 litters); and F_1 males born to control mothers were mated with F_1 females born to control mothers (*N* = 8 litters). b) The maternal (ML) and paternal lineages (PL) of F_1 poly(I:C) offspring for the subsequent generation of F_2 offspring was dissected. To obtain F_2 poly(I:C) offspring via the ML, female F_1 poly(I:C) offspring with male F_1 control offspring were crossed (*N* = 6 litters); and to generate F_2 poly(I:C) offspring via the PL, male F_1 poly(I:C) offspring with female F_1 control offspring were crossed (*N* = 7 litters). F_1 control males and F_1 control females were crossed to obtain the F_2 control lineage. c) Experimental flow chart: the prenatal infection was carried out with the mimetic molecule poly(I:C) to the F1 and F2 generations as shown in a) and b), subsequently the offspring was divided in two different cohorts, the first one on which the behavioral tests were applied, and the second one which continue with the Microbial sample collection from the cecum, the DNA extraction, the 16SrRNA library preparation and NGS with IM-Illumina MiSeq or IM-Ion Torrent PGM and finally the bioinformatics analysis and data processing with Qiime.

5.1 Poly(I:C) prenatal infection model. C57Bl6/N mice were used throughout the study. To generate the first-generation (F_1) offspring of poly(I:C)-exposed or control mothers (F_0), female mice were subjected to a timed-mating procedure. Pregnant F_0 dams on gestation day (GD) 9 were randomly assigned to receiving either a single injection of poly(I:C) (5 mg/kg) or vehicle (sterile pyrogen-free). For each experimental series involving F_0 exposures, a total of 16 pregnant dams were used, half of which were allocated to the poly(I:C) treatment, and the other half to the vehicle treatment.

The selected gestational window (GD 9) in mice corresponds roughly to the middle of the first trimester of human pregnancy with respect to developmental biology and percentage of gestation from mice to humans. All F_1 offspring (48 offspring) were weaned and sexed on postnatal day (PND) 21. Littermates of the same sex were caged separately and maintained in groups of 3 to 5 animals per cage. Upon reaching early adulthood (PND 70), F_1 offspring were either allocated to behavioral testing or breeding, the latter of which served to produce subsequent generations of immune-challenged or control ancestors. Hence, behaviorally naive littermates were always used as breeding pairs to obtain the F_2 generation, thereby avoiding possible confounds in breeding mice arising from prior behavioral testing. For generating the F_2, F_1 males born to poly(I:C)-exposed mothers were mated with F_1 females born to poly(I:C)-exposed mothers; and F_1 males born to control mothers were mated with F_1 females born to control mothers. In a second series of experiments, the maternal (ML) and paternal lineages (PL) of F_1 poly(I:C) offspring were dissected for the subsequent generation of F_2 offspring. To obtain F_2 poly(I:C) offspring via the ML, female F_1 poly(I:C) offspring was cross with male F_1 control offspring; and to generate F_2 poly(I:C) offspring via the PL, male F_1 poly(I:C) offspring was mate with female F_1 control offspring. F_1 control males and F_1 control females were crossed to obtain the F_2 control lineage[17].

5.2 Behavioral testing. For each generation, behavioral testing started when the offspring reached PND 70 and included tests assessing social interaction, cued Pavlovian fear conditioning, pre-pulse inhibition (PPI) of the acoustic startle reflex, and behavioral despair in the forced swim test. For each generation, 1-2 offspring per sex and litter were randomly selected and behaviorally tested to minimize possible

confounds arising from litter effects. Both male and female offspring were used in the first experimental series. Given that the first experimental series did not reveal sex-dependent effects in the F_1 and F_1 poly(I:C) offspring, all subsequent experimental series were conducted using male offspring only in order to minimize the number of animals. The sample sizes ranged from 9 to 14 offspring per group and sex[17].

It is important to mention that the poly(I:C) model and the behavioral testing were the ones of the previous research[17]: 'Transgenerational transmission and modification of pathological traits induced by prenatal immune activation'. We used these immune active and tested mice for our further NGS processing and analysis of gut microbiota relating the transmission of behavioral phenotypes across generations already reported with the possible dysbiosis in the gut microbiota which might work as a microbial vector that might be also horizontally transmitted across generations.

5.3 Microbiota sample collection. All the animals were sacrificed by decapitation to proceed with the sampling. Intestinal cecum from rodent was obtained (Figs 11&12).

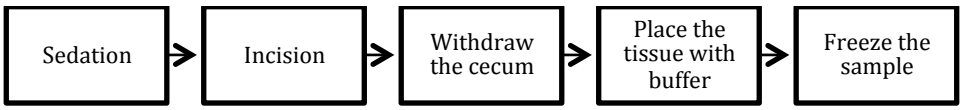


Figure 11. Sample collection process of cecum in mice (from left to right)

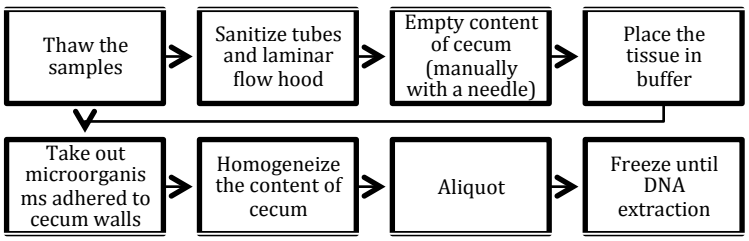


Figure 12. Acquire content (intestinal microbiota) from cecum process

Then the content from cecum was acquire, to obtain a sample of intestinal microbiota found in it (Figure 12). The microbiota sample collection was held by the Physiology and Behavior Laboratory, Swiss Federal Institute of Technology (ETH) Zurich, Schwerzenbach, Switzerland.

5.4 DNA extraction. The DNA extraction (Figure 13) from intestinal contents for further 16S libraries preparation was carried out with the DNA extraction kit: QIAmp DNA Stool Mini Kit[66].

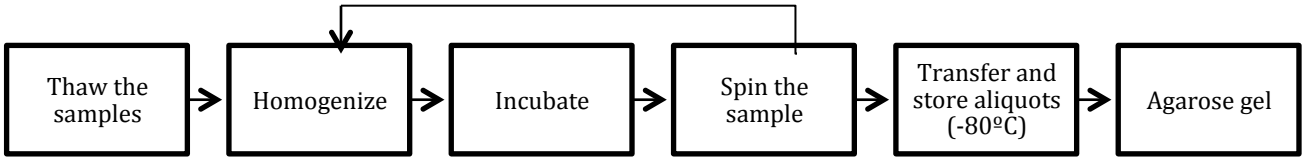


Figure 13. DNA extraction process from left to right

5.5 16S Library Preparation and Next generation sequencing.

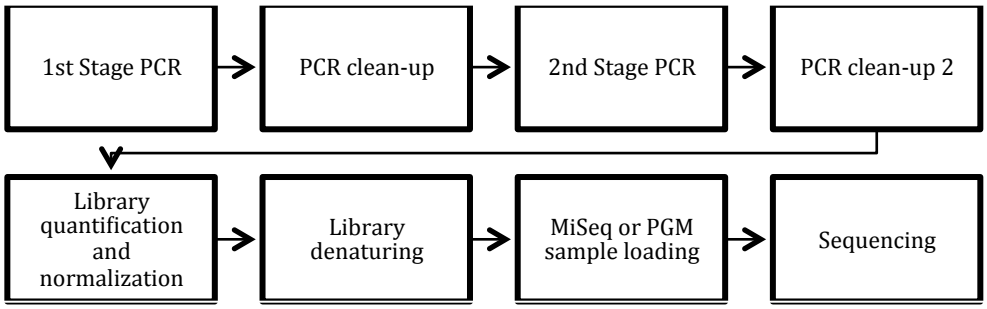


Figure 14. 16S Library preparation process for further sequencing

The F_1 samples were subjected to massive sequencing in two different types of next-generation sequencers: 1) *Ion Torrent PGM* at the Support Reference Laboratory for Characterization of Genomes, Transcriptomes and Microbiome of CINVESTAV (CINVESTAV–IPN Mexico), and 2) *Illumina MiSeq* at the Department of Chemistry,

Biotechnology and Food Science in Norwegian University of Life Sciences (NMBU-Norway). It is important to mention that with the exception of the second objective where we compared the results of two different NGS platforms (IM-Illumina Miseq vs. IT-Ion Torrent PGM), all the samples were sequenced for consistent results with only one platform (IM-Illumina MiSeq since this was carried out at NMBU). Both the library preparation and the next generation sequencing of the samples were carried out in the aforementioned laboratories.

5.6 Analysis and data processing. The analysis and data processing was divided in three main phases (Figure 15), each one represents one of the specific objectives.

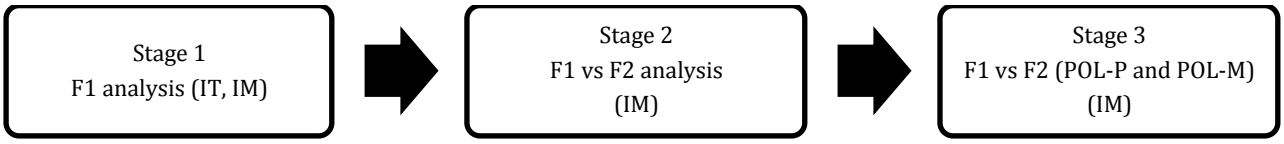


Figure 15. Three main phases (in order form left to right) of the analysis and data processing

This bioinformatic analysis and data processing obtained from the next generation sequencing was carried out using bioinformatic tools such as: Qiime, Useacrh, Uparse, Uchime, Trimommatic, FastQC, Matlab Clustal X and MEGA; additionally, Greengenes was used as the reference data base for all the analyzes. This processing is divided into 3 main stages or scripts: The Pre-processing, the OTU picking, and the Core diversity analysis (Table 3).

Table 3. Three stages or scripts (Pre-processing, OTU_picking and Core diversity) used for the bioinformatic processing of the data with their function, commands and bioinformatic tools.

Stage (script)	Function	Commands	Bioinformatic tools
Pre-processing	Decompressing files, extract barcodes, join forward and	'Extract_all_barcodes.py' 'Join_all_pairedends.py' 'Split_all_libraries.py'	Qiime Trimmomatic FastQC

	reverse reads, trimming, quality assessment, and merge sequences into a large sequence file.	'fastq_stats' 'fastq_filter' 'Add_qiime_labels.py'	USEARCH
OTU_picking	Taxonomic assignment, inference of phylogeny, creation of an OTU table.	'Derep_full_length' 'Abundance sort' 'OTUs de novo' 'Reference chimera check' 'Assign_taxonomy.py' 'Filter alignment'	Qiime USEARCH UPARSE UCHIME
Core_diversity	Alpha and beta diversity, comparisons of samples, taxonomy graphs, statistical analysis of significance between groups.	'Alpha_rarefaction.py' 'Beta_diversity_through_plots.py' 'Summarize_taxa_through_plots.py' 'Compare_alpha_diversity.py' 'Group_significance'.	Qiime

Since the F_1 samples were processed with two different sequencers (Illumina Miseq and Ion Torrent PGM), some modifications to the Pre-processing script were carried out. The following stages: the OTU picking and the Taxonomical assignment and diversity were run identically regardless the platform.

5.6.1 Pre-processing (Script1 'Filtering')

The preprocessing includes a series of steps and commands that prepare our data merging all sequences into one large sequence file, decompress all files, get the quality statistics and in the case of the *IM* samples extract the barcodes and join all forward and reverse reads.

All processes need a metadata file or mapping file; it must be created with all the information of our samples. As a minimum, must contain the barcode sequence used for each sample, the name of the samples, the sequence primer used to amplify the sample and a description column[44]. This document must be created with raw text format

as a .txt. Subsequently we must ensure that the mapping files previously generated, have a properly formatted and are free of errors that may affect the analysis. Qiime contains a command that performs this function

5.6.1.1 Pre-processing with IM-Illumina MiSeq samples

1) The Pre-processing with the IM files starts decompressing the files generated by the sequencer.
2) Then, the barcodes must be extracted, using the *'Extract_All_barcodes.py'* command, which is designed to a fastq sequence format and barcode data. In the output directory, there will be fastq files (barcode file, and one or two reads files)[66]. We extract the barcodes in each of our 48 samples of F_1 and 42 samples for the F_2.
3) Next, forward and reverse IM reads need to be joined with the '*Join_All_pairedends.py'*. This script takes forward and reverse IM reads and joins them using the method chosen. Will optionally create an updated index reads file containing index reads for the surviving joined paired end reads[66]. The 'Join paired_ends' method (default method) has been selected for the samples.
4) The next step is splitting all libraries; we demultiplex fastq sequence data. In this step, we are "turning off" filter parameters, and storing the demultiplexed fastq file with the *'Split_All_Libraries.py'* command (Qiime scripts)
5) Then, all the sequences are merged into one large sequence file. This file will be used in the second stage or script: the OTU_picking.

6) After that, the quality statistics that are needed for the trimming and quality assessment for the following steps are obtained. The '*fastq_stast'* command is used, which reports statistics on reads in a fastq file[67].
7) Finally, the quality reads must be removed. We make a trimming of the sequences, and a quality assessment with the values obtained in the log file of the previous step, using the *'fastq_filter'* command, which performs this quality filtering and trimming of the sequences, and also the conversion of a fastq file to fasta format[67].

A value should be selected for the option *–fastq_maxee E,* which discard reads with > E total expected errors for all bases in the read after any truncation options have been applied, in both analyzes (F_1 and F_2), we selected a value of 0.33; for the *–fastq_minlen* L option, which deletes sequences with < L number of basepairs, as the variable regions V3 and V4 were sequenced with IM for both generations (~450 pb), a min length of 350pb was selected.

5.6.1.2 Pre-processing IT-Ion Torrent PGM samples.

Since for the IT samples we only work with forward reads and not with reverse reads, and the sequences are free of barcodes and decompressed by the sequencer, we don't need to use the commands for this.

1) First is the trimming and quality assessment of the samples. Here, we evaluate the quality by base sequence and quality scores, the content basis, the distribution of sequence length and presence and quality of adapters, in order to crop, adjust, or remove sequences.

We chose the command line of Trimommatic and FastQC as a graphical tool to carry out this first step of pre-processing the F_1 samples with IT. Since the variable region V3 of the 16SrRNA gene was assessed (with length of 250pb) the sequences with less than 150bp and a quality score phred33 were discarded, also both sides were trimmed by removing bases that not displayed in a range of good quality for each of the 48 samples. It is noteworthy that each of the samples contains a range of 25,000 to 260,000 sequences per sample.

2) Next, the data format of the files must be adapted to be processed with Qiime. IT sequencer, generates the data in a fastq or SFF format, so it must be converted to a fasta format (accepted format by Qiime).
3) The last step is to generate one single file (*seqs.fna*), which contains all the information and sequences of each of our samples merging them all. The *add_qiime_labels.py* command is used, which takes a directory, a metadata mapping file, and a column name that contains the Fasta file names that SamplesIDs are associated with, combines all the files that have valid Fasta extensions into a single Fasta file, with valid Qiime Fasta labels[66]. A '*combined_seqs.fasta'* file will be created in the output directory, with the sequences assigned to the SampleID given in the metadata-mapping file[44].

Since the merged file containing all the sequences and information of the samples is generated in both Pre-processing analyses we can continue with the OTU picking.

5.6.2 OTU selection (Script 2 'OTU_picking')

The OTU picking, is possibly the most important stage taken, is where the taxonomic assignment, the inference of phylogeny and the creation of an OTU table takes place. Obtaining Operational Taxonomic Units (OTUs) is based on the similarity of the sequences within the readings, and one representative sequence of each OTU is obtained. The protocol, assigns taxonomic identities using a reference database, aligns the OTU sequences, creates a phylogenetic tree and builds an OTU table, which represents the abundance of each OTU in every sample. This protocol requires a demultiplexed sequences file as those generated in the previous step in both pre-processing analyzes. In this stage other bioinformatics tools such as Usearch, Uchime and Uparse are used. This second stage 'OTU_picking' in turn is divided into three stages: Filtering processing, OTU processing and Taxonomy processing.

5.6.2.1 Filtering processing

1) The first step: 'derep_full_length', discard duplicated sequences, annotate with cluster sizes and sort by decreasing cluster size. The aim is to reduce the number of readings with errors. The *–sizeout* option may be used to specify that size annotations are added to the unique sequence labels. USEARCH supports full-length and prefix dereplication, but currently not substring[67].
2) The next step: 'Abundance sort', is used for clustering when more abundant sequences make better centroids. In 16S OTUs, more abundant sequences are likely to be accurate biological sequences while rare or singleton reads are more likely to contain sequencing errors or be due to PCR artifacts such as chimeras[67].
3) The 'sort_by_size' command, sort sequences by decreasing size annotation, which usually refers to the size of a cluster. The size is specified by a field -*size*=N; where

N is an integer. The -*minsize* option can be used to specify a minimum size. In this case it was decided to use a –*minsize* of 2, so readings containing a single sequence will be discarded as being consider chimeras or artifacts.

5.6.2.2 OTU processing

1) OTU Clustering (*de novo* chimera checking). The UCLUST algorithm divides a set of sequences into clusters. A cluster is defined by one sequence, known as the centroid or representative sequence. Every sequence in the cluster must have similarity above a given identity threshold with the centroid. UCLUST is effective at identities ~75% above for nucleotides. The *cluster_otus* command performs this OTUs grouping using the algorithm UPARSE-OTU[67]. A threshold of 97% of similarity between sequences is assigned to form part of the same cluster.
2) Subsequently the 'reference chimera check' is performed. The fundamental step in UCHIME is a search for a 3-way alignment of a query sequence with two parent sequences (A and B) such that one parent is more similar to one segment of the query (Q) and the other parent is similar over another segment. A score is calculated from the alignment. Higher scores indicate a stronger chimeric signal. A score cutoff set by the -minh option (0.28 by default) determines whether the query is classified as a chimera. This search can be performed with a reference database provided by the user, or the database can be constructed de novo from the query sequences. For the *uchime_ref* command, the reference database should include sequences that might appear as parents in the query set. These should be high-quality sequences that are believed to be free of chimeras. Errors in reference sequences

will degrade detection accuracy and increase the number of false positives. Chimeras will not be detected if their parents (or sufficiently close relatives) are not present in the database[67].

3) In the next step, the 'fasta_number.py' command, will replace fasta labels with *xxx*1, *xxx*2, *xxx*3 etc, where *xxx* is a prefix provided as a command-line argument. Used e.g. to label OTUs as OTU_1, OTU_2 etc[67].
4) After that the 'Usearch_global' command is used, to search for one (default) or a few high-identity hits to a database using the USEARCH algorithm. The alignments are global. To get more than one hit, increase *–maxaccepts*, an identity threshold must be specified. Fasta and UDB formats are supported[67]. A threshold of 97% of identity and Greengenes database were selected.
5) Next, the OTU_table will be generated with help of the 'uc2otutab_mod.py' command. This OTU_table is a .txt file that contains all the information obtained from our OTUs sequences.
6) Finally, this OTU_table is converted in a biom format, so that can be carried out to further analysis (taxonomy assignment).

5.6.2.3 Taxonomy processing

1) Taxonomy assignment. Given a set of sequences, 'assign_taxonomy.py 'attempts to assign the taxonomy of each sequence. The output of this step is an observation metadata-mapping file of input sequence identifiers (1st column of output file) to taxonomy (2nd column) and quality score (3rd column). There may be method-specific information in subsequent columns[66]. Greengenes database was selected.

2) Add taxonomy to biom file. This taxonomic assignment now will be added to the biom file (OTU_table.biom) generated in previous steps, creating a new OTU_table, which will become again in a txt format for easy viewing and analysis.
3) Subsequently the alignment of sequences is performed in Qiime, with the 'align_seqs.py' command, which aligns the sequences in a FASTA file to each other or to a template sequence alignment, depending on the method chosen. All aligners will output a fasta file containing the alignment and log file in the directory specified by --*output_dir*. Greengenes database was used[66].
4) Next, the 'Filter alignment' command is used, this script should be applied to generate a useful tree when aligning against a template alignment. Additionally, the user can supply a lane mask file, that defines which positions should be included when building the tree, and which should be ignored[66].
5) Then, the tree is built with the 'make_phylogeny.py' command, which produces a tree from a multiple sequence alignment[66].
6) Finally, a compilation is generated in a summary of all the information that resides in the biom file. A BIOM file is taken as input, and print a summary of the count information on a per-sample basis including the number of sequences and readings, the number of observations and the total count (sum of all values in the table) to the new file specified by the -o parameter[66].

5.6.3 Core diversity analysis (Script3 'Core_diversity')

This last stage of all this processing consists in a script that plugs several Qiime diversity analyzes together to form a basic workflow beginning with a biom table,

mapping file, and optional phylogenetic tree. The included scripts are those run by the workflow scripts[66]:

- *alpha_rarefaction.py*, generates rarefied OTU tables, computes alpha diversity metrics for each rarefied OTU table, collate alpha diversity results and generate alpha rarefaction plots for each index that the user selects.
- *beta_diversity_through_plots.py*, performs beta diversity, principal coordinate analysis, and generate a preferences file along with 3D PCoA Plots.
- *summarize_taxa_through_plots.py*, summarizes OTU by category, summarize taxonomy and plot taxonomy summary at the different phylogenetic levels.
- *compare_alpha_diversity.py*, compares the alpha diversity of samples found in a collated alpha diversity file. The comparison is done not between samples, but between groups of samples. The groupings are created via the input category.
- *group_significance.py*, is used to compare OTU frequencies in sample groups and to ascertain whether or not there are statistically significant differences between the OTU abundance in the different sample groups. The script will compare each OTU based on the passed sample groupings to see if it is differentially represented. The sample groupings are determined by the -c option. Any samples that do not contain a value under the given header will not be included in the comparison. At a basic level, the script is constructing an OTUxSample (rowXcolumn) contingency table, and testing whether or not each OTU is differentially represented in certain groups of columns (determined by the metadata category passed).

Is important to mention that here the depth of sequencing is selected, the normalization value or values to be set for the sequences of each of the samples (standardization). For both analyzes (IT and IM) first the value of normalization of the sequences per sample was selected. Three values for the IT F_1 were chosen: 5,000, 7,000 and 10,000 and two for the IM $F_1$and F_2: 13,000 and 15,000. This because of the different amount of sequences per sample that each analysis present.

In each of the standardization analyzes, a different number of samples is discarded. Those samples that are discarded are the ones that do not reach the set number of sequences per sample. These values were chosen randomly, trying to make the smallest possible number of discarded samples. For the alpha analysis: *Shannon, Simpson, Simpson reciprocal* and *Chao1* indexes were selected, also the number of *Observed Species*. Finding significant differences between groups was conducted with the statistical analysis for nonparametric data, Kruskal Wallis. A p value ≤ 0.05 was consider as significant. In addition to this three stages of data processing, for all the analyzes with both generations F_1 and F_2 some *MATLAB* plots of the significant OTUs were created, and taxonomy trees were build using the RDP (Ribosomal Database Project), MEGA and Clustal X for a better analysis of the data.

6. Results and Discussion

6.1 F_1 generation gut microbiota sequenced with IT-Ion Torrent PGM vs. IM-Illumina MiSeq.

Is important to mention that with the exception of mandatory differences in platform-specific sequencing adaptors, the bidirectional sequencing strategy used in IM (forward and reverse primers) and the hypervariable regions that were sequenced (V3 IT vs. V3 and V4 for IM) which necessarily are uncontrolled variables, the processing and data analysis of the samples was maintained identically for both sequencing NGS technologies.

The generated data with two NGS platforms presents several similarities and some important differences. This 16S rRNA amplicon analysis was done by separating the data in the two interest groups poly(I:C) (POL) and control (SAL) for each NGS platform analysis, focusing on the finding of similarities and differences between both; specially in the abundance and the presence of bacterial communities in each group that can lead to a dysbiosis.

The normalization of the number of sequences per sample was better for the analysis with IM (13,000 and 15,000 sequences/sample) since most of the samples were found in a smaller range of sequences, in contrast to the IT samples (5,000, 7,000 and 10,000 sequences/sample).

6.1.1 Taxonomy classification. The taxonomy classification plots (Figs 16 & 17) shows the most abundant bacterial communities that were present in each group. We

did not find significant difference (Chi^2) in taxonomy between the diverse analyzes of standardization neither on IM nor in IT.

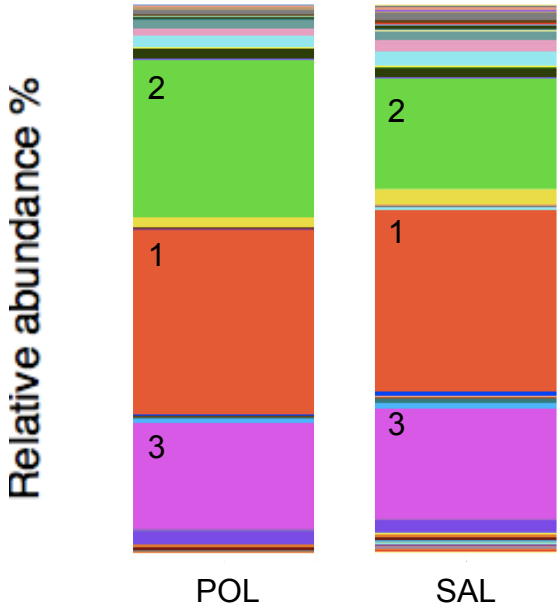


Figure 16 F1 I**T-I**on Torrent taxonomy assignment (10,000 normalization value) in both treatment groups (POL: poly(I:C) offspring, SAL: control offspring)**.** A genus of the order of Clostridiales (1) as the most abundant community with ~30%, a genus of the family of Lachnospiraceae (2) as the second most abundant community with ~25%, and a genus of the S24-7 family (3) as the third most abundant community with ~20%.

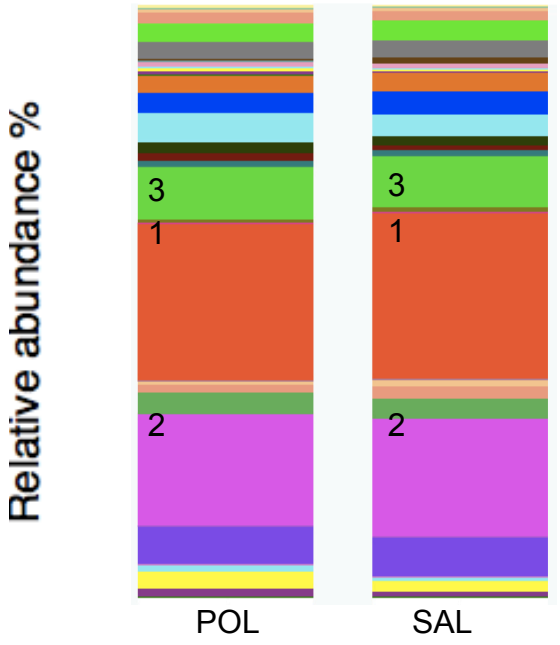


Figure 17 F_1 IM-Illumina taxonomy assignment (15,000 normalization value) in both treatment groups (POL: poly(I:C) offspring, SAL: control offspring). A genus of the order of Clostridiales (1) as the most abundant community

with ~26%, a genus of the family S24-7 (2) as the second most abundant community with ~19%, and a genus of the family of Lachnospiraceae (3) as the third most abundant community.

In the F_1 IT samples showed a dominance of the bacterial communities *Firmicutes* and *Bacteroidetes* can be observed; a genus of the order of *Clostridiales* as the most abundant community with ~30% in both groups and a genus of the family of *Lachnospiraceae* as the second most abundant community with 28.5% in the POL group and a less abundance in the SAL group with 20.0% both of them from phylum *Firmicutes*. From the *Bacteroidetes* phylum a genus of the *S24-7* family is the third most abundant community with ~20%.

Now using IM platform the F_1 data indicate also a dominance of bacterial groups *Firmicutes* and *Bacteroidetes*. Being the most abundant bacterial community the genus of the order of *Clostridiales* with ~26% in both groups. A genus of the family *S24-7* from the *Bacteroidetes* phylum was the second most abundant community with ~19%, and a genus of the family *Lachnospiraceae* was the third most abundant community with 8.9% in the POL group and 8.6% in the SAL group. It should be noted that a reduced relative abundance in both treatment groups was observed compared with the IT taxonomy results where this community was the second most abundant with 28.5%, and 20.0% respectively.

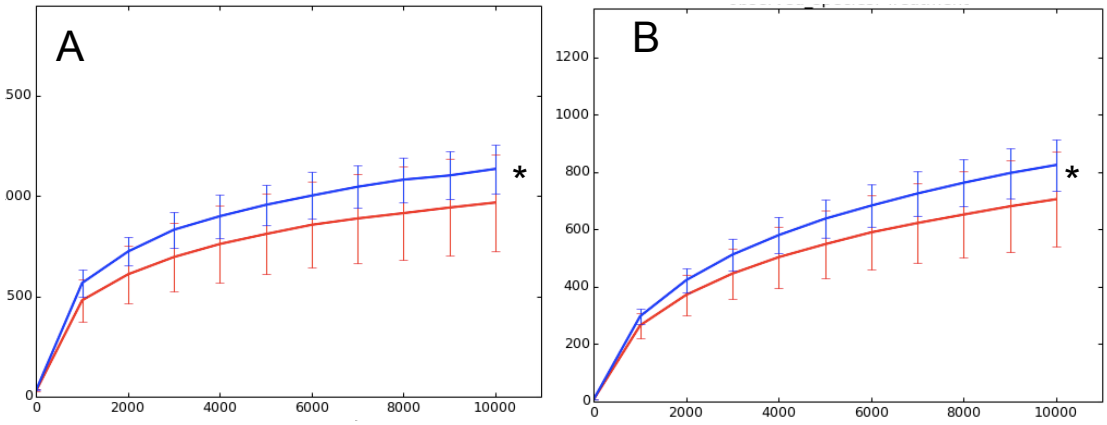
For both sequencing technologies (IT vs. IM), a very similar distribution of the most abundant bacterial communities was obtained the groups, with a predominance of the order *Clostridiales* from the phylum *Firmicutes* and the family *S24-7* order of *Bacteroidales* and from the *Bacteroidetes* phylum. Hoffman et al. [3] reported results with

similarities in the taxonomy abundance of bacterial communities when comparing both sequencers (IT vs. IM).

6.1.2 Alpha diversity analysis. Rarefaction curves are shown in Figures 18 & 19 of the 10,000 and 15,000 sequences/sample normalization values respectively are presented as they implicitly include the other standardization analysis (5,000, 7,000 IT and 13,000 sequences/sample IM, plots can be visualized in the Annex 2).

For the IT case in all the rarefaction curves it was observed a tendency and a significant difference of the POL group to have lower diversity values in contrast to the SAL group. This result can be interpreted as a less diversity and abundance of bacterial species in the POL group in comparison to the SAL group. For the IM case, no significant difference (t-stud) was observed, as they don't present more or less abundance with respect the other group. This data is contradictory since for IT NGS platform there is a significant group effect, where as for the IM NGS platform groups have a similar abundance of bacterial diversity regardless of pre-natal treatment (POL vs. SAL).

6.1.2.1 Rarefaction plots



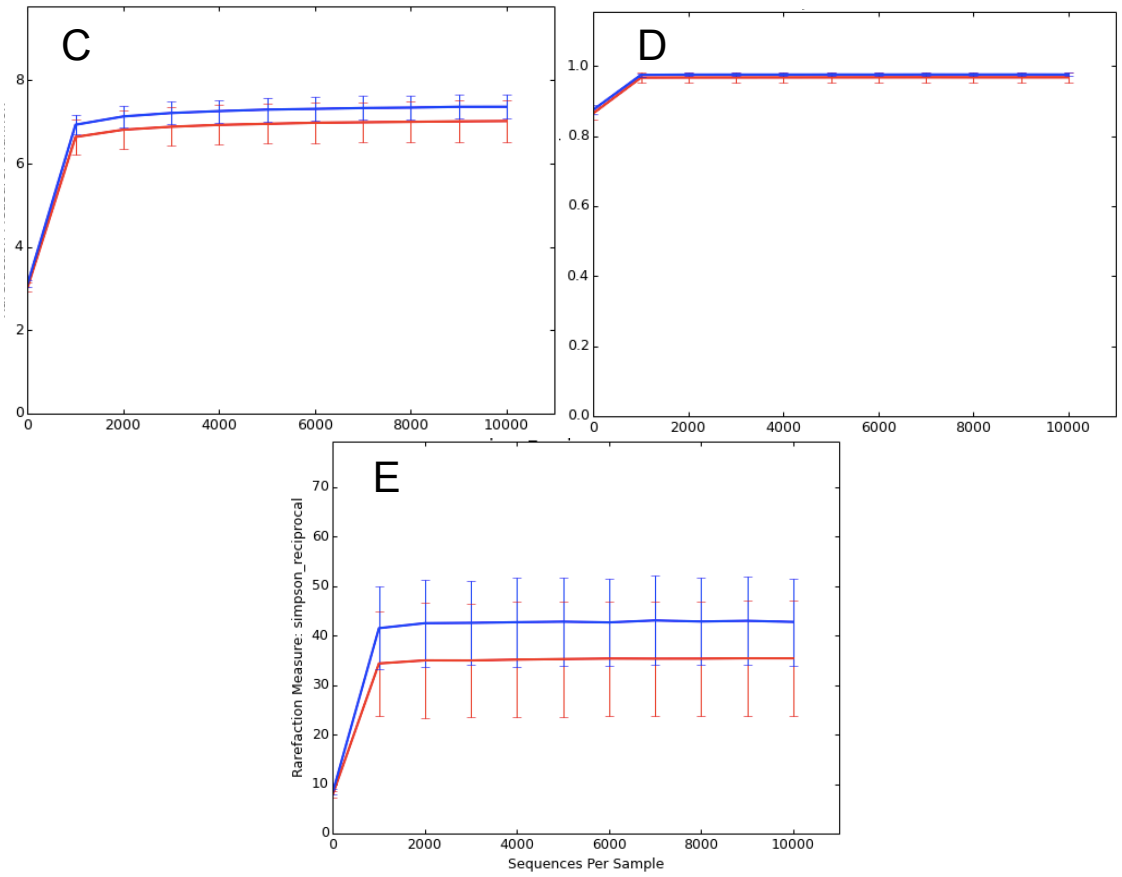
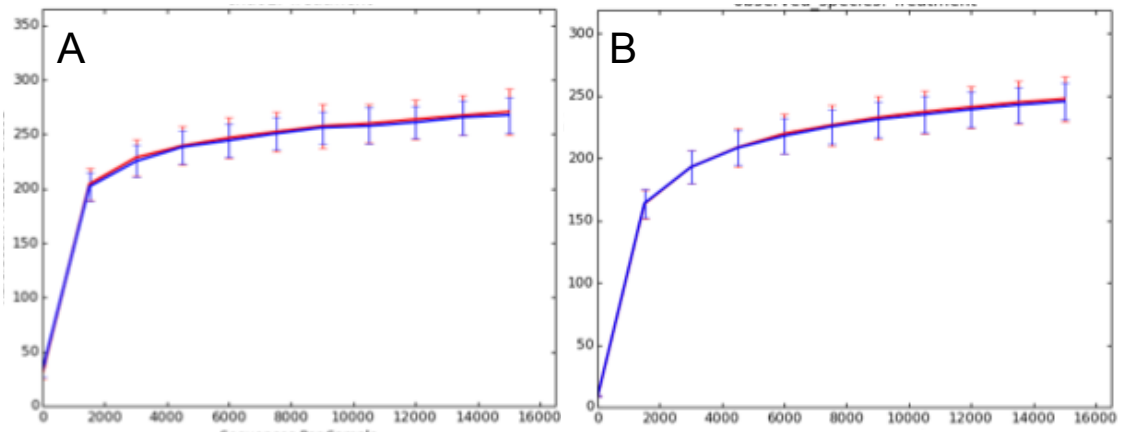


Figure 18. F1 Rarefaction curves of alpha diversity in both treatment groups (POL: poly(I:C) and SAL: control) with the Ion Torrent analysis. Indexes of diversity: A) Chao1, B) Observed species, C) Shannon, D) Simpson and E) Simpson reciprocal. **POL-red line**, **SAL-blue line**. A tendency of less diversity in the POL group with respect the SAL group can be observed.



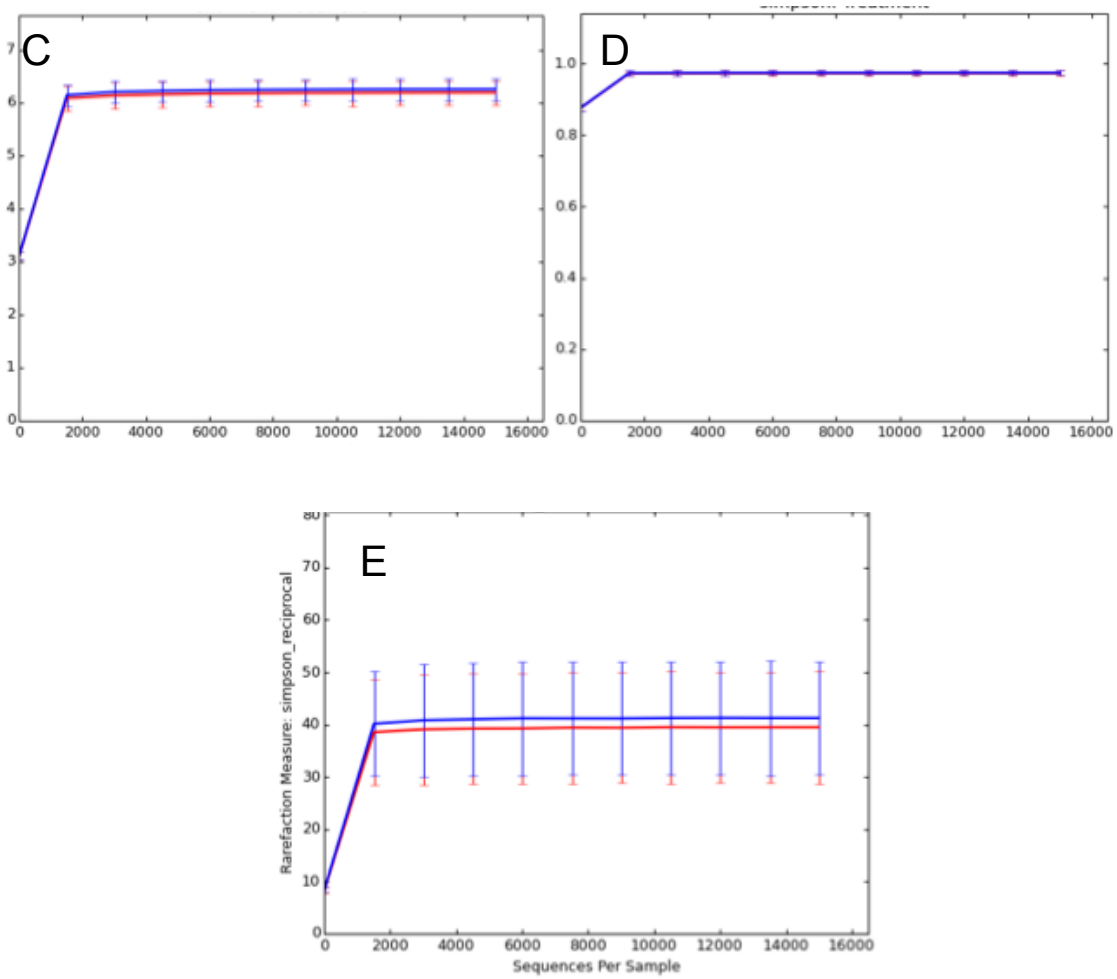


Figure 19. F1 Rarefaction curves of alpha diversity in both treatment groups (POL: poly(I:C) and SAL: control) with the Illumina analysis. Indexes of diversity: A) Chao1, B) Observed species, C) Shannon, D) Simpson and E) Simpson reciprocal. POL-red line, SAL-blue line. No tendency of less or more diversity can be found in any of the treatment groups.

It is relevant to mention that in both cases in Shannon, Simpson and Simpson reciprocal indexes can be observed that the sequencing effort was enough, as the curve tends to stabilize before the 2,000 sequences/ sample.

6.1.2..2 Statistic analysis of alpha diversity. The statistical analysis (t-stud) for the IT samples the Chao 1 index (Fig 18-A) and the Observed species (Fig 18-B) reported a statistical significant difference between both groups F_1 POL and F_1 SAL, in all the different analyzes of standardization. This data can be interpreted as the diversity and abundance in the F_1 POL samples were less than those in the F_1 SAL samples.

Interestingly, in the other three indexes Shannon (Fig 18-C), Simpson (Fig 18-D) and Simpson reciprocal (Fig 18-E) there wasn't a statistical significant difference, but the mean values obtained showed that there is a trend of lower diversity of the F_1 POL group compared to the F_1 SAL group. In contrast, for the IM data the rarefaction curves showed no statistical significant difference (t-stud) between the abundance and diversity of the F_1 POL and F_1 SAL groups (Fig 19).

In this regard, alpha diversity analysis showed controversial results when comparing two different NGS platforms, as the analysis with the IT platform did found statistically significant differences, whereas IM did not. A possible explanation for this inconsistence might be related to some artifacts in sequencing or an error rate of sequencers. It is important to mention that to our knowledge differences in alpha diversity comparing two or more NGS platforms have not been previously reported, thus the validity and reproducibility of this sequencing technologies might be still questionable, and the necessity of standardization procedures and references are necessary and desirable to develop in the near future.

6.1.3 Significant OTU selection. Interestingly, a different quantity of significant OTUs were obtained for each of the standardization values in both analyzes IT vs. IM (Tables 4 & 5). The complete tables with all the significant OTUs could be visualized in Annex3.

Table 4. Number of significant OTUs with the IT-Ion Torrent analysis in the three normalization values (5000, 7000 and 10,000 sequences/sample)

5,000 Analysis	7,000 Analysis	10,000 Analysis
77 Significant OTUs	88 Significant OTUs	100 Significant OTUs

Table 5. Number of significant OTUs with the IM-Illumina analysis in the two normalization values (13,000 and 15,000 sequences/sample)

13,000 Analysis	15,000 Analysis
15 Significant OTUs	10 Significant OTUs

The total assignment of bacterial communities in each of our analysis, generated a different value of OTUs: 411 in IM and 2,756 in IT. This may explain why a different amount of significant OTUs (Kruskal Wallis) was obtained from each of the treatment groups depending on the NGS platform; for IT 100 statistically significant OTUs were obtained, while for IM just 10 statistically significant OTUs were obtained (both statistical analyzes were performed taking into account the larger value of: 1,000 and 15,000 sequences per sample respectively). It is important to mention that we found common significant OTUs independent of the NGS platform employed. For instance, the *S24-7* families from the *Bacteroidetes* phylum, diverse OTUs from the order of Clostridiales, some Prevotella genus and Distasonis species. However, the reported relative abundance of these OTUs varied considerably between the NGS platforms. Similarly, Hoffman et al.[3] have previously reported that the abundance of one or more organisms detected by a NGS platform (OTUs) was significantly different from that detected by other NGS platform.

Most of the significant OTUs found in the IT analysis belong to the *Firmicutes* and *Bacteroidetes* phylum:

- Different orders of *Clostridiales* and *Lachnospiraceae, Ruminococcaceae* and *Erysipelotrichaceae* families from *Firmicutes*

- Many *S24-7* and some *Rickenellaceae* families, some *Prevotella* and *Oridobacter* genus from the *Bacteroidetes* phylum.
- Also significant bacterial communities present from the *Proteobacteria* phylum: *Rhodobacter, Sutterella* and *Rhodoplanes* genus, and *Chromatiaceae* and *Alcaligenaceae* families.

Consistentetly, most of the significant OTUs found in the IM analysis also belong to the *Firmicutes* and *Bacteroidetes* phylum:

- From the *Firmicutes* phylum: orders of *Clostridiales,* families of *Lachnospiraceae* and *Ruminococcaceae*, and *Candidatus Arthromitus, Dehalobacterium* and *Dorea* genus are present.
- From the *Bacteroidete*s phylum: *S24-7* and *Rikenellaceae* families, *Prevotella* and *Parabacteroides* genus
- One genus from *Actinobacteria* phylum: *Adlercreutzia*.

6.1.3.1 Significant OTU selection regardless the normalization value. It is important to mention that not all of the statistically significant OTUs found in one analysis with a specific NGS platform / normalization value, were found with another analysis with a different NGS platform / normalization value (Figs 20 & 21). Therefore, we decided to focus just on the OTUs that remained constantly statistically significant different between treatment groups regardless the value of normalization.

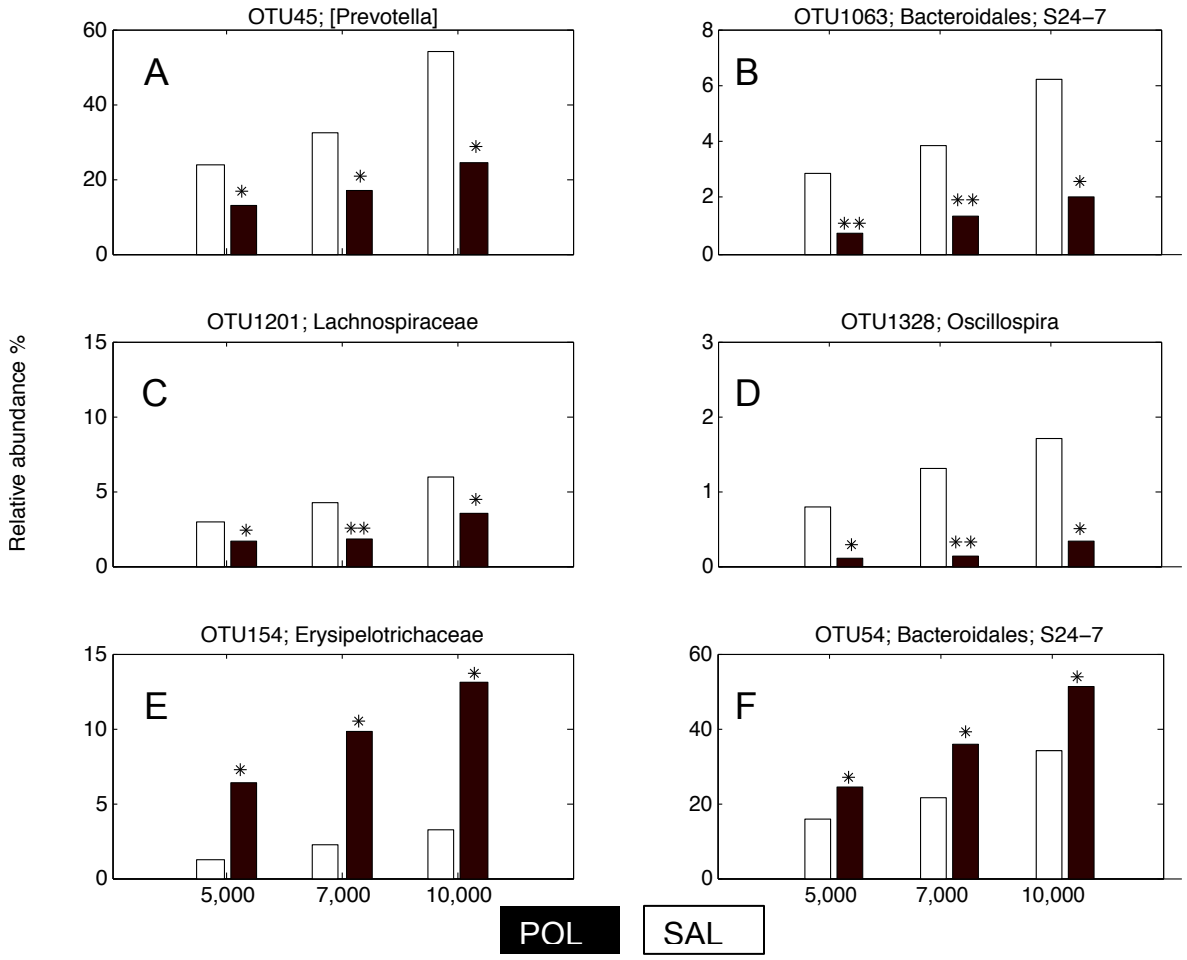


Figure 20. 6 OTUs that show statistically significant difference between groups. White: SAL (control group), Black: POL (poly(I:C)) group, regardless the normalization value with IT-Ion Torrent analysis.
A-D show OTUs that are less abundant in the POL in comparison to SAL (*Bacteroidales S24-7, Prevotella, Oscillospira, Lachnospiraceae*); E-F show OTUs that are more abundant in the POL in comparison to SAL (*Bacteroidales S24-7 and Erysipelotrichaceae*).

For the IT analysis, 6 OTUs that remained statistically significant different (Kruskal Wallis) in the three analyzes were obtained (5,000, 7,000 and 10,000 sequences/sample), 3 from the phylum of the *Bacteroidetes* (the genus *Prevotella* of the family *Paraprevotellaceae* (Fig 20 A) and 2 genera of the family *S24-7* of the order *Bacteroidales* (Fig 20 B and Fig 20 F) as well as 3 OTUs of the phylum *Firmicutes* (the family *Lachnospiraceae* of the order *Clostridiales* (Fig 20 C), the genus *Oscillospira* of the family *Ruminococcaceae* (Fig 20 D) and the family *Erysipelotrichaceae* of the order *Erysipelotrichales* (Fig 20 E). It must be taken into account, that the OTUs 45

(*Prevotella*), 1063 (*S24-7*), 1201 (*Lachnospiraceae*) and 1328 (*Oscillospira*) showed a significantly less abundance in the POL group with respect the SAL treatment (Fig 20 A-D). Conversely the OTUs 54 (*S24-7*) and 154 (*Erysipelotrichaceae*) showed a greater abundance in the POL with respect the SAL group (Fig 20 E-F).

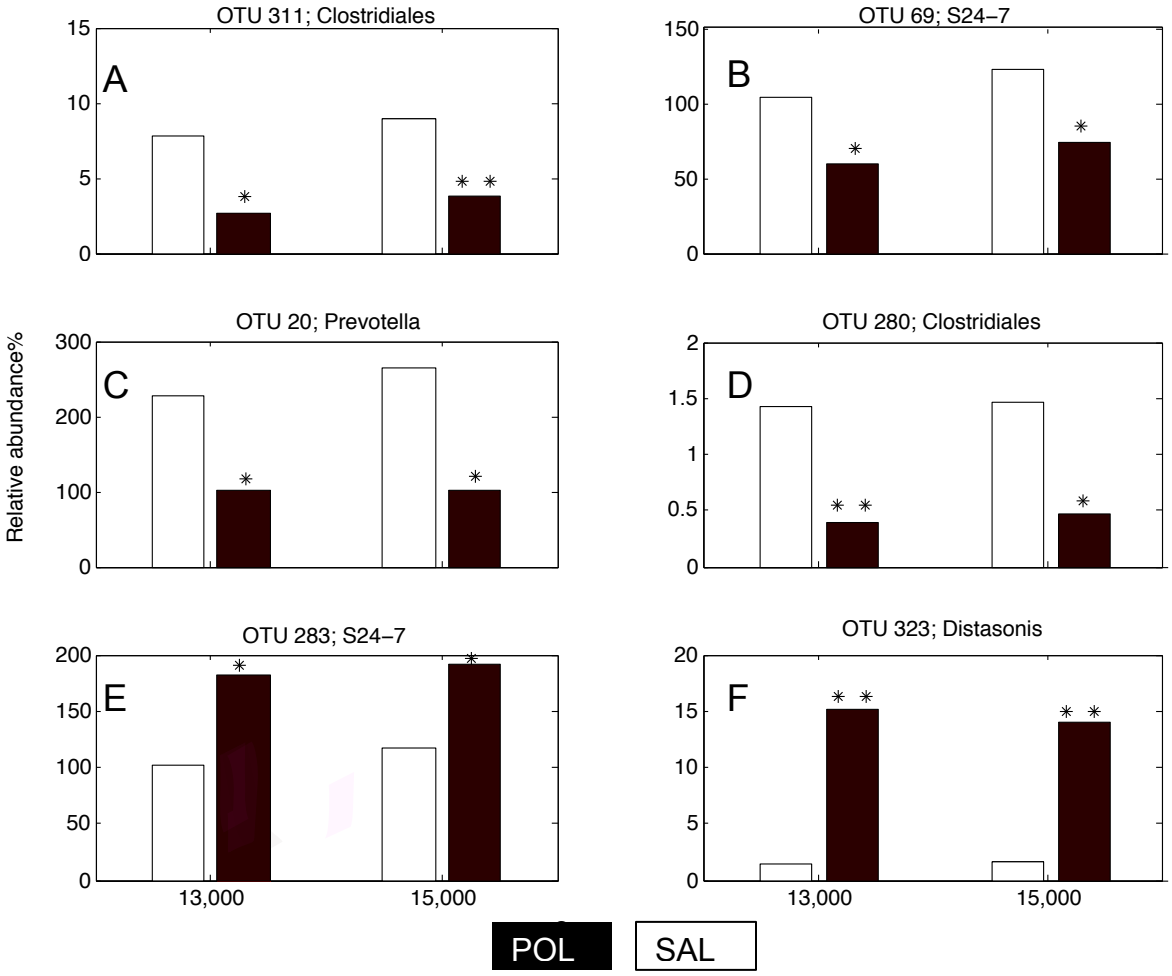


Figure 21. 6 OTUs that show significant difference between groups White: SAL (control group), Black: POL (poly(I:C)) group, regardless the normalization value with Illumina analysis.
The first 4 OTUs (Clostridiales, Bacteroidales S24-7, Prevotella) are less abundant in the POL group, the last 2 OTUs (Bacteroidales S24-7 and Distasonis) are more abundant in the POL group.

For the IM analysis, also 6 OTUs that remained significant (Kruskal Wallis) after of normalization (13,000 and 15,000 sequences/sample); 4 from the phylum of the *Bacteroidetes:* the genus *Prevotella* of the family *Paraprevotellaceae* (Fig 21 C), two genus of the family *S24-7* of the order *Bacteroidales* (Fig 21 B and Fig 21 E) and the

specie *Distasonis* of the genus *Parabacteroides* (Fig 21 F) as well as 2 OTUs of the phylum *Firmicutes*: two families of the order *Clostridiales* (Fig 21 A & D). In this case, the OTUs 323 (*Distasonis*), and 283 (*S24-7)* showed a significantly higher abundance in the POL group; conversely the OTUs 69 (*S24-7*), 280 (*Clostridiales*), 20 (*Prevotella*) and 311 (*Clostridiales*) showed a less abundance in the POL group in comparison to SAL condition (Fig 21 A-D).

6.1.4 Phylogenetic analysis. For a better visualization and classification of these 12 significant OTUs, phylogenetic trees were built using Clustal X, MEGA, and a taxonomy reassignment with Ribosomal Database Project (RDP).

As shown in the phylogenetic tree data is organized into 2 microbial clusters; one that belongs to the phylum *Bacteroidetes* and the other belonging to the phylum *Firmicutes* (Figure 22). From the *Bacteroidetes* cluster a closer grouping showed three pairs of OTUs; the OTUs 20 IM and 45 IT remained grouped and seemed that they belong to species of *Prevotella,* being both of them less abundant in the F_1 POL group (Fig 20 A & Fig 21 C). The OTUs 283 and 69 IM and 54 and 1063 IT cluster together belonging to the order *Bacteroidales* as in the Greengenes assignment, but in a more specific way they seemed to be *Barnesiella* species. It is remarkable that OUT 69-IM and OUT 1063-IT showed a less abundance in the F_1 POL group (Fig 21 B & Fig 20 B), and the other pair (OTU 283-IM and OUT 54-IT) showed a dominance of abundance in the F_1 POL group with respect the F_1 SAL group (Fig 20 F & Fig 21 E).

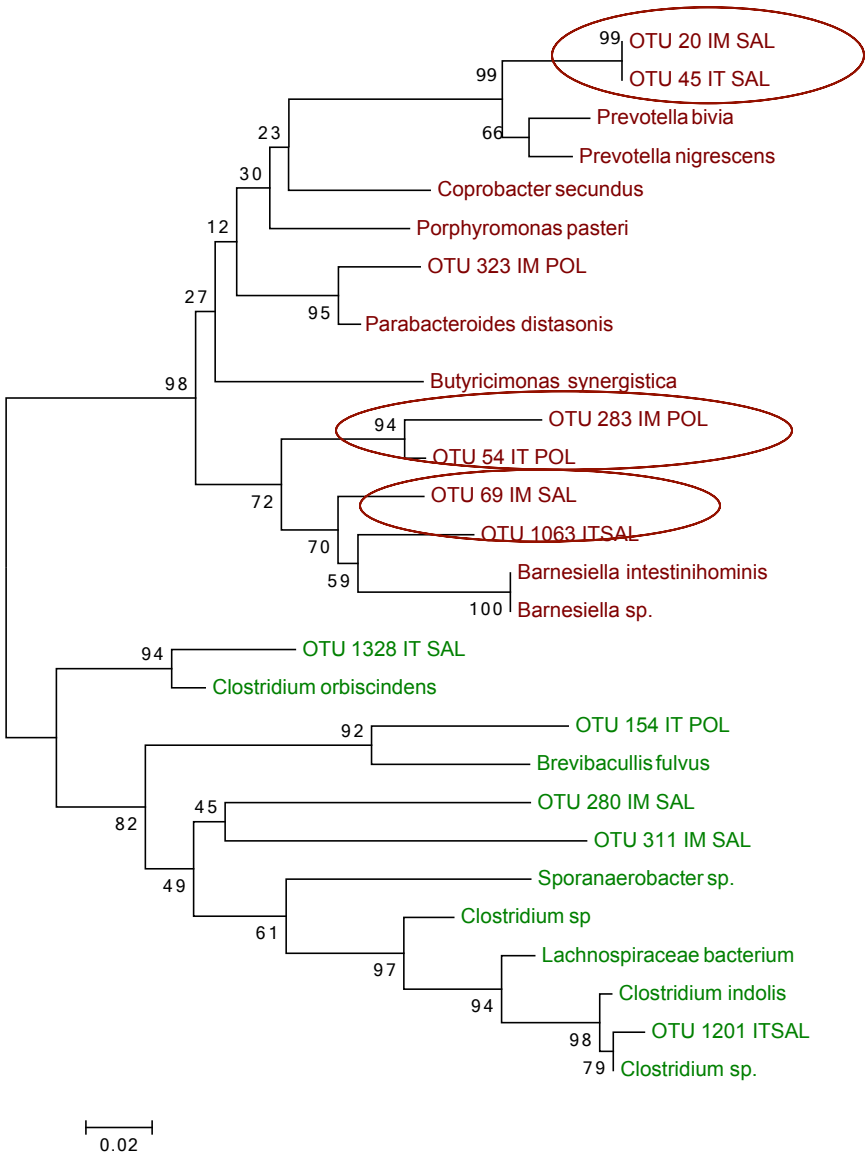


Figure 22. Phylogenetic tree of the significant OTUs in the F1 generation for both analyzes (IT-Ion Torrent and IM-Illumina). (The numbers in each branch are the values of the Bootstrap and the length of the branches the phylogenetic distance) Firmicutes phylum: Green / Bacteroidetes phylum: red. Taxonomy re-assignment with RDP. Prevotella OTUs (20 IM and 45 IT) and Barnesiella OTUs (283 and 69 IM, 54 and 1063 IT), are present as significant in both NGS platforms.

In the other big cluster (*Firmicutes*) OTUs were not grouped with a high similarity as in the first one. All OTUs seemed to be some species and genus of *Clostridiales*, but there was not a specific grouping between OTUs with any of the NGS platforms.

With the help of the phylogenetic tree and the generated clusters it is documented that some of these significant OTUs in each analysis are phylogenetically close, with some of them being reassigned taxonomically using the RDP database.

In summary, these results indicate that there are differences in the presence and abundance of bacterial communities in the gut microbiota between the F_1 generation of immune challenged mice that present a neurodevelopmental disorder generated by poly(I:C) with respect the control mice. This change might be considered as an irregularity in the microbiota within the body and therefore a dysbiosis. Importantly, we found important and significant differences in results with each type of NGS platforms as shown in Table 6. However, results from both NGS platforms (IM or IT) consistently reported the irregularity in the gut microbiota with specific bacterial communities of the orders *Clostridiales* and *Bacteroidales*.

Table 6. Summary and comparison between platforms (IT and IM). * Normalization value (sequences/sample), o_order, f_family, g_genus, s_specie.

Specifications	IT-Ion Torrent PGM	IM-Illumina MiSeq
Sequenced hypervariable regions	V3	V3 and V4
Normalization values	5,000 7,000 and 10,000	13,000 and 15,000
Taxonomy assignment (most abundant bacterial communities)	Order Clostridiales Family Lachnospiraceae Order Bacteroidales (S24-7)	Order Clostridiales Order Bacteroidales (S24-7) Family Lachnospiraceae
Alpha diversity	Less diversity abundance in POL group	No difference in diversity abundance between groups
Total OTU selection	2,756 OTUs	411 OTUs
Significant OTUs between treatment groups POL vs SAL	5,000*= 77 OTUs 7,000*= 88 OTUs 10,000*= 100 OTUs	13,000*= 15 OTUs 15,000*= 10 OTUs
Significant OTUs between treatment groups POL vs SAL regardless the normalization value	g_Prevotella, 2 o_Bacteroidales (S24-7), f_Lachnospiraceae, g_Oscillospira, f_Erysipelotrichaceae.	2 o_Clostridiales, 2 o_Bacteroidales (S24-7), g_Prevotella, s_Distasonis.
More abundant significant OTUs in POL group	f_Erysipelotrichaceae and o_ Bacteroidales (S24-7)	O_ Bacteroidales (S24-7) and s_Distasonis
Less abundant significant OTUs in POL group	g_Prevotella, 2 o_Bacteroidales (S24-7), f_Lachnospiraceae, g_Oscillospira.	2 o_Clostridiales, o_Bacteroidales (S24-7), g_Prevotella.

6.2 Possible transmission of bacterial communities that are causing dysbiosis between the F_1 to the F_2 generation.

For the second objective the transmission of a dysbiosis in the gut microbiota from the F_1 to the F_2 generation was assessed. The same way as in the first objective, the analysis was done by separating the data in the two treatment groups POL and SAL but now also analyzing it across two generations (F_1 vs. F_2). It is important to mention that just IM-NGS platform was employed to avoid an additional variable. All the F_1 generation analysis is the same data that was already obtained in the first objective (IM analysis). Additionally, the value of normalization of 15,000 sequences/sample was selected based on the previous results; all the subsequent analyzes were reported using it. Plots with data of the 13,000 sequences/sample normalization value can be observed in Annex2.

6.2.1 Taxonomy classification. Taxonomy remained very similar in both generations as shown in Figs 23 & 24. The same bacterial communities appeared to be the most abundant and a dominant: *Firmicutes* and *Bacteroidetes*.

The most abundant bacterial community was a genus of the order of *Clostridiales* with ~28% in both generations, and it seemed to be more abundant in the F_2 generation with 26.3% (F_1-POL) and 29.4% (F_2-POL) and 27.8% (F_1-SAL) and 31.1% (F_2-SAL). A genus of the family *S24-7* from the *Bacteroidetes* phylum was the second most abundant community with ~19% presenting a little more abundance in the F_2 generation POL group 18.8% (F_1) and 19.63% (F_2). A genus of the family of *Lachnospiraceae* was the third most abundant community with less abundance in the F_2 generation with 8.9%

(F_1-POL) and 5.1% (F_2-POL) and 8.6% (F_1-SAL) and 7.5% (F_2-SAL). A genus of the family of *Rumminococcaceae* presented more abundance in both POL and SAL groups for the F_2 generation with 5.0% (F_1-POL) and 6.0% (F_2-POL), and 3.6% (F_1-SAL) and 5.8% (F_2-SAL).

By the other way a genus of the family of *Rikenellaceae* presented less abundance in both POL and SAL groups for the F_2 generation with 6.5% (F_1-POL) and 3.7% (F_2-POL) and 6.8% (F_1-SAL) and 3.6% (F_2-SAL). No significant difference neither in the SAL groups or POL groups between generations was found (Chi^2).

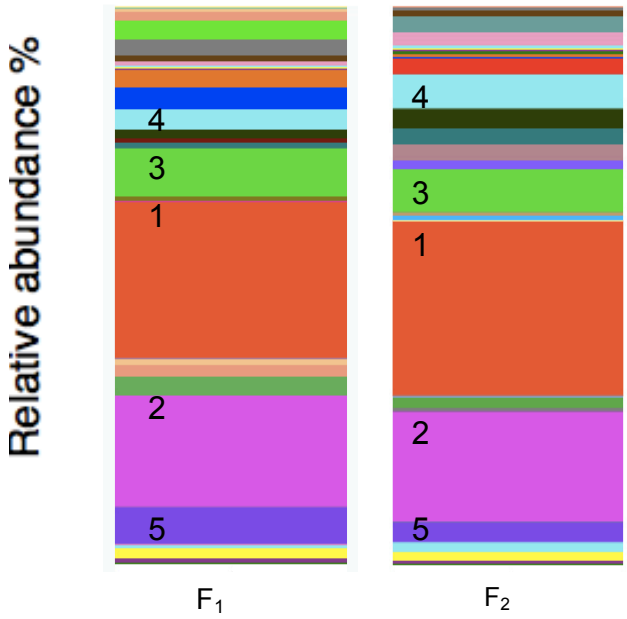


Figure 23. Taxonomy assignment for the F_1 and F_2 generations in SAL: control offspring treatment group. A genus of the order of *Clostridiales* (1) with 27.8% (F_1-SAL) and 31.1% (F_2-SAL), a genus of the family *S24-7* (2) with 19.7% (F_1-SAL) and 19.5% (F_2-SAL), a genus of the family of *Lachnospiraceae* (3) with. 8.6% (F_1-SAL) and 7.5% (F_2-SAL), a genus of the family of *Rumminococcaceae* (4) with 3.6% (F_1-SAL) and 5.8% (F_2-SAL) and a genus of the family of *Rikenellaceae* (5) with 6.8% (F_1-SAL) and 3.6% (F_2-SAL).

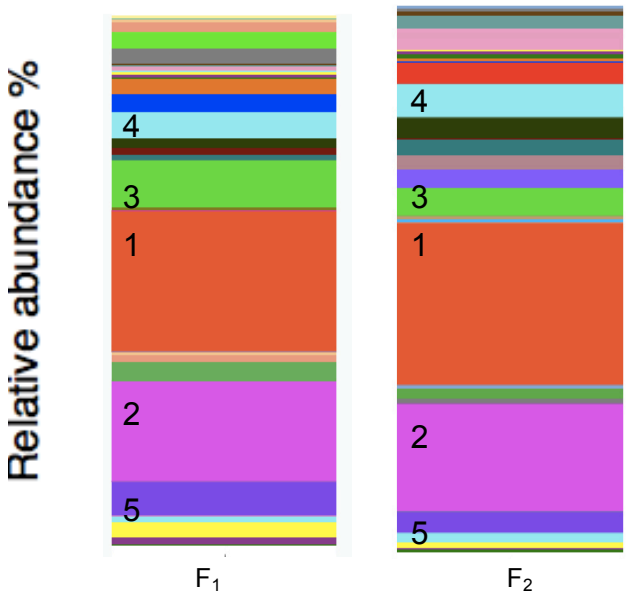


Figure 24. Taxonomy assignment for the F1 and F_2 generations in POL: poly(I:C) offspring treatment group. A genus of he order of *Clostridiales* (1) with 26.3% (F_1-POL) and 29.4% (F_2-POL), a genus of the family *S24-7* (2) with 18.8% (F_1-POL) and 19.63% (F_2-POL), a genus of the family of *Lachnospiraceae* (3) with. 8.9% (F_1-POL) and 5.1% (F_2-POL), a genus of the family of *Rumminococcaceae* (4) with 5.0% (F_1-POL) and 6.0% (F_2-POL) and a genus of the family of *Rikenellaceae* (5) with 5.0% (F_1-POL) and 6.0% (F_2-POL).

6.2.2 Alpha diversity analysis. For the alpha diversity analysis, in both cases no significant group difference of abundance in the alpha diversity rarefaction plots (Annex 2) were observed. This data allow us to conclude that both treatment groups remain with a very similar diversity for each generation F_1 and F_2. Consistently, no statistically significant difference were found in abundance and diversity between the POL and SAL groups in both generations F_1 and F_2 with the statistical analysis (data not shown).

6.2.3 Significant OTU selection. The number of total obtained assigned OTUs in each analysis was different. The F_1 generation had an allocation of 411 OTUs and the F_2 generation an allocation of 342 OTUs before applying the statistical test (Kruskal Wallis) for significance. The number of statistically significant OTUs between each of the generations was different for each generation: 10 significant OTUs were obtained in the F_1 generation and 30 significant OTUs in the F_2 generation. It is noteworthy that the vast

majority of these OTUs belong to the same families and genera across generations. The complete tables with all the significant OTUs could be visualized in Annex3.

6.2.3.1 Significant OTU selection F_1. For the F_1 analysis all statistically significant OTUs belong to the *Firmicutes* and *Bacteroidetes* phylum, as follow:

- Different genus from the order of *Clostridiales,* and the *Mogibacteriaceae* family also from the *Clostridiales* order.
- Many *S24-7* families, the *Prevotella* genus and the *Distasonis* specie from the *Bacteroidales* order.

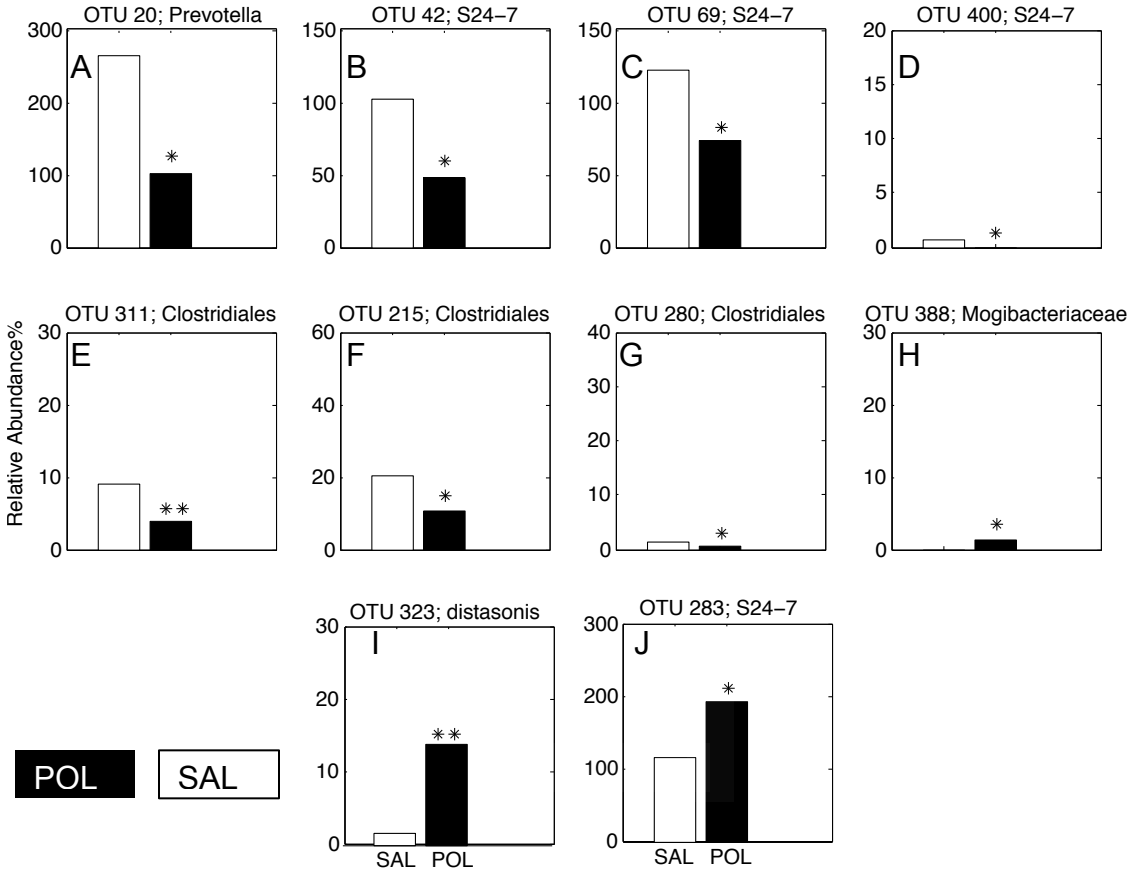


Figure 25. Significant OTUs present between treatment groups (POL: poly(I:C) and SAL: control) in the F_1 generation. A-G show OTUs that are less abundant in the POL group than in SAL (Clostridiales, Bacteroidales S24-

7, Prevotella); H-J show OTUs are more abundant in the POL group than in SAL (Mogibacteriaceae, Bacteroidales S24-7 and Distasonis).

Analysis indicate that for the F_1 generation the OTUs 20 (*Prevotella)*, 42 (*S24-7*), 69 (*S24-7*), 400 (*S24-7*), 311 (*Clostridiales*), 215 (*Clostridiales*) and 280 (*Clostridiales*) showed a significant less abundance in the POL group than in the SAL group (Fig 25 A-G), and in the case of the OTUs 323 (*Distasonis*), 283 (*S24-7*) and 388 (Mogibacteriaceae) they showed more abundance in the POL group than SAL group (Fig 25 H-I).

This F_1 generation results that we obtained show some similarities with the previously reported Hsiao[35] results, were they postulated that "the microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders" with the same poly(I:C) model in a F_1 generation offspring; they reported changes in the diversity of the gut microbiota specifically in *Clostridia* and *Bacteroidia* OTUs (same as we are reporting), however, in the next taxonomic levels (family, genus or specie) they found as significant the bacterial communities of the families: *Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae, Alcaligenaceae, Byphyromonadaceae, Prevotellaceae, Rikenellaceae* and *un-classified Bacteroidales,* where only the *Prevotellaceae* family coincided with our results. Interestingly, they reported that *Porphyromonadaceae*, *Prevotellaceae*, unclassified Bacteriodales and *Lachnospiriceae* were more abundant in poly(I:C) offspring. Conversely, *Ruminococcaceae*, *Erysipelotrichaceae*, and the beta *Proteobacteria* family *Alcaligenaceae* were more abundant in control offspring, suggesting that specific *Lachnospiraceae*, along with other *Bacteroidial* species, may play a role in ASD pathogenesis, while other taxa may be protective. And we reported that *Mogibacteriaceae*, *Bacteroidales* S24-7 and

Distasonis were more abundant in the poly(I:C) offspring and *Clostridiales*, another *Bacteroidales* S24-7 and *Prevotella* were more abundant in the control group, suggesting that *Mogibacteriaceae* and *Distasonis* may play a role in ASD pathogenesis, while *Prevotella* and another *Clostridiales* may be protective.

In another previous research of gut microbiota in ASD human individuals, Song et al.[68], reported a significant increase specifically in *Clostridium bolteae* and *Clostridium* clusters I & X, and Wang et al.[69] reported higher abundance of *Sutterella* and *Ruminococcus* spp. in individuals with ASD compared to controls without a family history of ASD.

6.2.3.2 Significant OTU selection F_2. In the F_2 generation, most of the significant OTUs between treatment groups POL and SAL belong to the *Firmicutes* and *Bacteroidetes* phylum and some others belong to the *Cyanobacteria* and *Verrucomicrobia* phylum, as follows:

- Several genus of the order *Clostridiales,* the *Mogibacteriaceae, Lachnospiraceae* and *Ruminococcaceae* families, and the *Ruminococcus, Lactobacillus* and *Oscillospira* genus and the *gnavus* specie from the *Clostridiales* order.
- Two *S24-7* families, and two *Rikenellaceae* families from the *Bacteroidales* order.
- The order *YS2* from the *Cyanobacteria* phylum.
- The specie *Muciniphila* order of *Verrucomicrobiales* from the *Verrucomicrobia* phylum.

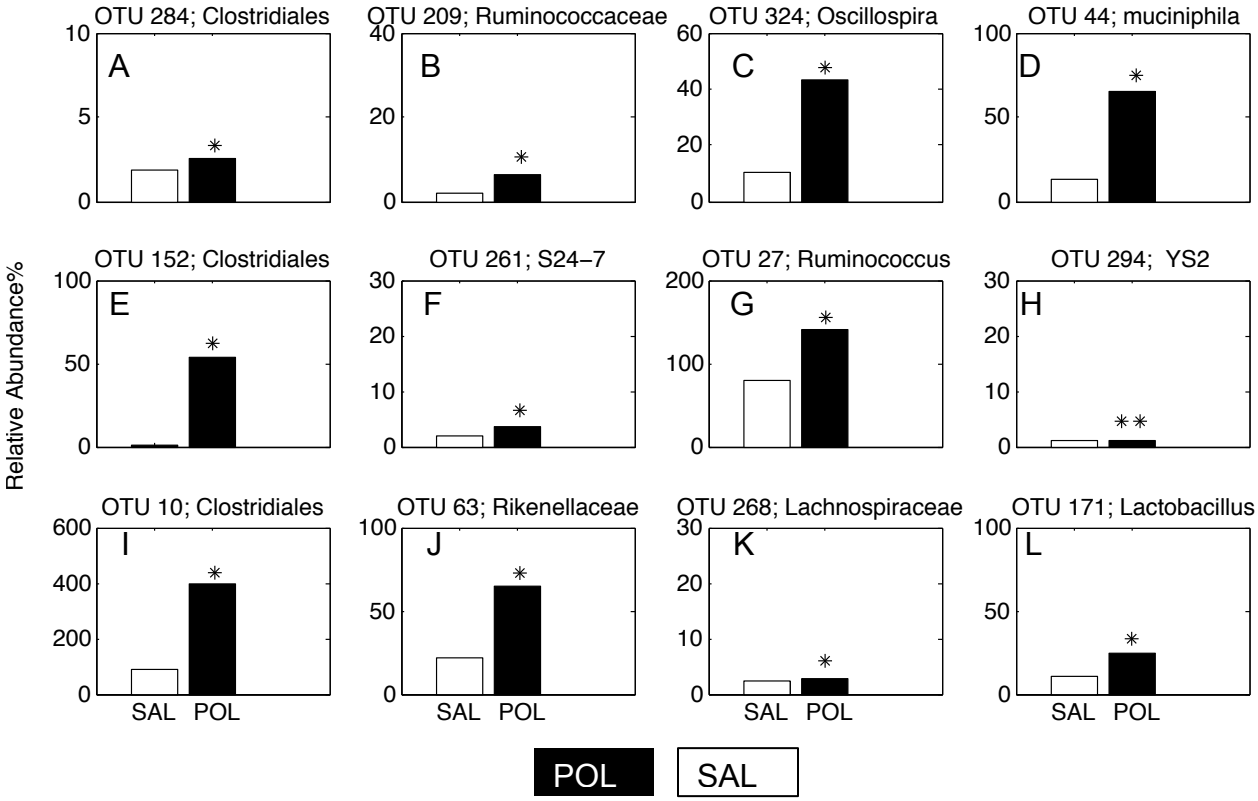


Figure 26. Significant OTUs present between treatment groups (POL: poly(I:C) and SAL: control) in the F_2 generation, this OTUs presented more abundance in the POL group.

As shown in Figure 26 the OTUs: 284 Clostridiales (A), 209 Ruminococcaceae (B), 324 *Oscillospira (C), 44 Muciniphila (D), 152 Clostridiales (E), 261 S24-7 (F), 27 Ruminococcus (G), 294 YS2 (H), 10 Clostridiales (I), 63 Rikenellaceae (J), 268 Lachnospiraceae (K*) and *171 Lactobacillus (L*), showed more significant abundance in the F_2 POL group with respect the F_2 SAL group. By the other way in Figure 27 the OTUs: *301 Oscillospira (A), 84 Clostridiales (B), 129 Oscillospira (C), 191 Ruminococcus (D), 91 Oscillospira (E), 180 S24-7 (F), 45 Clostridiales (G), 252 Mogibacteriaceae (H), 76 Clostridiales (I), 343 Ruminococcus (J), 153 Ruminococcaceae (K), 39 Clostridiales (L), 248 Ruminococcus (M), 246 Clostridiales (N), 293 Lachnospiraceae (O), 183 Rikenellaceae (P) and 263 Clostridiales (Q)* showed less significant abundance in the F_2 POL group with respect the F_2 SAL group.

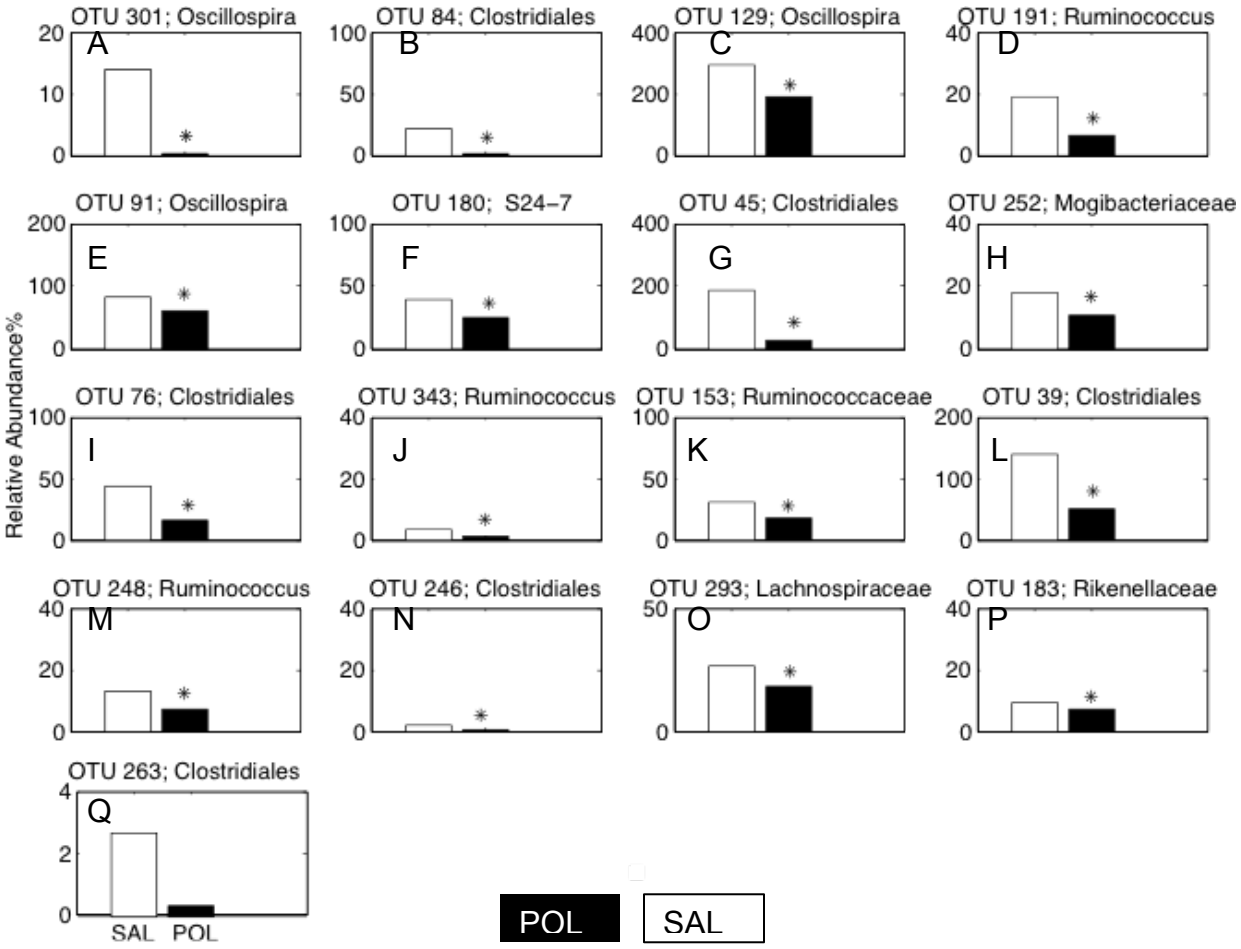


Figure 27. Significant OTUs present between treatment groups (POL: poly(I:C) and SAL: control) in the F_2 generation, this OTUs presented less abundance in the POL group.

Furthermore, for the F_2 generation, some of the significant OTUs between treatment groups from the F_1 generation remained present (i.e. transmitted): *S24-7* families, various orders of *Clostridiales* and the *Mogibacteriaceae* family. However, some of the OTUs in the F_2 generation that are significant (Kruskal Wallis) between treatment groups were not found as statistically significant (Kruskal Wallis) in the F_1 generation, as follow: *Lachnospiraceae* and *Ruminococcaceae* families from the *Firmicutes* phylum, *Rikenellaceae* families from the *Bacteroidetes* phylum, an order of *Cyanobacteria* and *Muciniphila* species from the *Verrucomicrobia* phylum (Figs 26 A-L & 27 A-Q).

Interestingly, in both F_1 and F_2 generations, most of the found significant OTUs were reported with a less abundance in POL groups compared to SAL groups.

6.2.4 Phylogenetic analysis. In the phylogenetic tree shown in Figure 28, it can be visualized that there are two big clusters of bacteria; the large one of the *Firmicutes* phylum and the small one of the *Bacteroidetes* phylum.

In the *Firmicutes* phylum cluster the pairs of OTUs: 215 F_1 with 263 F_2 and 280 F_1 with 246 F_2 from the order *Clostridiales* more specifically *Gracillibacter* species, produced differences in the gut microbiota all of them with less abundance in the POL group and more specifically were clearly transmitted from F_1 generation to F_2 generation.

On the other hand, within the *Bacteroidetes* phylum cluster presented a possible transmission from the F_1 to the F_2 generation as shown by the aggrupation of OTUs: 42, 283, 69 and 400 for the $F_{1,}$ and 261 and 180 for the $F_{2,}$ which were assigned as *Barnesiella* species. However, it is not clearly shown a relationship between the transmission of these significant OTUs across generations and their abundance in the POL group vs. SAL grup.

In summary, these results suggest that both F_1 and F_2 generations showed differences in the presence and abundance of bacterial communities in the gut microbiota of immune challenged mice that present a neurodevelopmental disorder generated by poly(I:C) with respect the control mice. The orders *Clostridiales* and *Bacteroidales* are confirmed as the primary drivers of gut microbiota differences between the prenatal F_1 treatment groups (POL vs. SAL). It is important to mention that specifically the species *Gracillibacter* (*Clostridiales*) with a less abundance in the POL group and *Barnesiella*

(*Bacteroidales)* were transmitted from F_1 generation to F_2 generation. All these results have a direct relationship with the results of novel phenotypes across generations[17] in which our work is based and the same cohorts of mice are analyzed, inasmuch as they demonstrated that prenatal viral-like immune activation leads to a transmission of behavioral phenotypes across generations. Whereas social interaction deficits and increased cued fear expression are similarly present in the F_1 and F_2 offspring of immune-challenged mothers, and increased behavioral despair emerges as a novel phenotype in the F_2 generation, suggesting that maternal immune activation during pregnancy can induce latent behavioral symptoms that are passed on to and become manifest only in subsequent generations.

In both researches, for the F_1 generation, the behavior changes (impaired sociability, increased fear expression, robust reduction un sensorimotor gating) and bacterial composition of gut microbiota (10 different significant OTUs mainly of the orders *Clostridiales* and *Bacteroidales)* was presented in the immune challenged mice (POL) in contrast to the control mice (SAL). Furthermore, for the F_2 generation these responses are increased, taking into account that there was a rise in the behavioral despair (similar deficit in sociability than F_1, increased fear expression, did not inherit the sensorimotor gating deficit, by contrast with the F_1 offspring that present an absence of behavioral despair associated with depressive-like behaviors, the F_2 offspring develop signs of behavioral despair) and therefore, we obtained a greater number of significant OTUs (17 OTUs also mostly from the orders *Clostridiales* and *Bacteroidales*) that present a different abundance in the gut microbiota of POL and SAL groups.

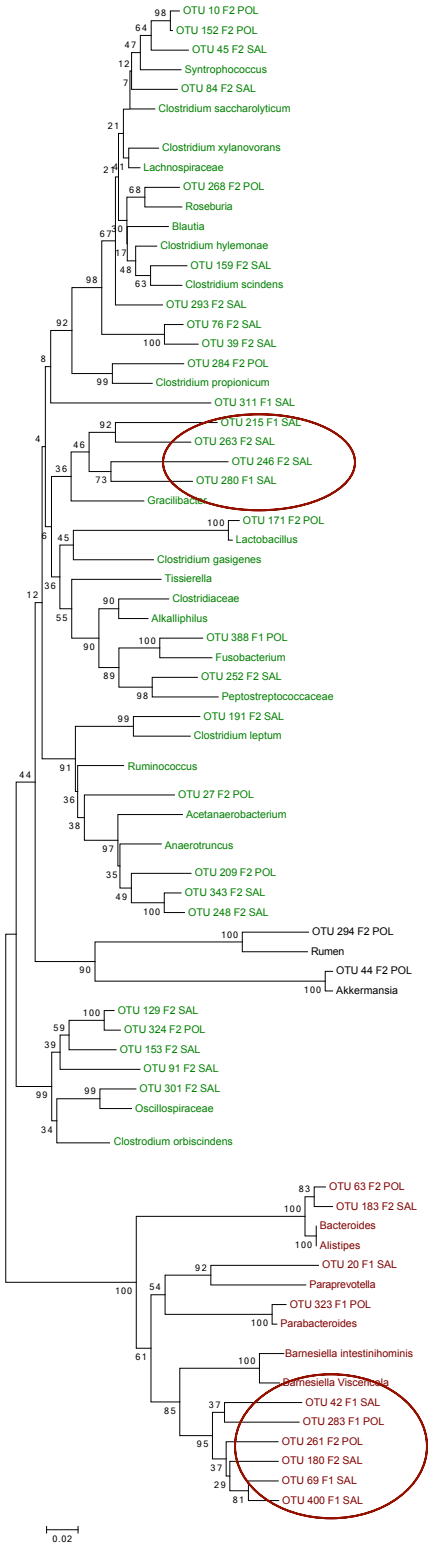


Figure 27. Phylogenetic tree of the significant OTUs transmitted from the first F_1 to the F_2 generation. (The numbers in each branch are the values of the Bootstrap and the length of the branches the phylogenetic distance) *Firmicutes* phylum: Green / Bacteroidetes phylum: Red / other phyla: black. The bacterial community *Gracillibacter* of significant OTUs (215 and 280 F_1, 263 and 246 F_2) is being transmitted from the F1 generation to the **F_2**. The set of *Barnesiella* OTUs (42, 283, 69 and 400 F_1 with 261 and 180 F_2) are also present in the dysbiosis of both generations.

6.3 Possible transmission of bacterial communities that are causing microbiota dysbiosis between the F_1 generation to the F_2 generation divided by progenitor lineages (POL-Maternal vs. POL-Paternal).

The third and last objective was to evaluate whether there is a progenitor linage (maternal: POL-M or paternal: POL-P) that is preponderant in the transmission of bacterial communities from the F_1 to the F_2 generation. The importance of this analysis is that the F_2 POL group was separated in two different categories depending on the progenitor lineage by which the offspring was generated maternal (POL-M) or paternal (POL-P). In the same way as in the previous results, this analysis used a 15,000 sequences/sample value for normalization. Additional analysis with other normalization can be found in Annex2.

6.3.1 Taxonomy classification. For the taxonomy assignment the three groups showed a very similar bacterial composition as shown in Figure 29. No significant difference neither in the SAL groups or POL groups between generations was found (Chi^2). The most abundant bacterial community in each case was a genus of the order of *Clostridiales* with ~32% (SAL and POL-M) and it seemed to be a little less diversity in the POL-P group with 26.0%, the second most abundant community was a genus of the *S24-7* family of phylum *Bacteroidetes* with a very similar percentage of abundance in the groups with ~20%, and the third most abundant bacterial community in each case was a genus of the family of *Lachnospiraceae* which showed to have less abundance in both POL groups 5.2% (POL-M) and 4.9% (POL-P) with respect 7.5% (SAL). The *Oscillospira* genus with 6.5% (POL-M), 5.6% (POL-P) and 5.8% (SAL) was also present

as an abundant bacterial community, the same as a genus of the *Rikenellaceae* family with 3.9% (POL-M) 3.6% (POL-P) and 3.6% (SAL).

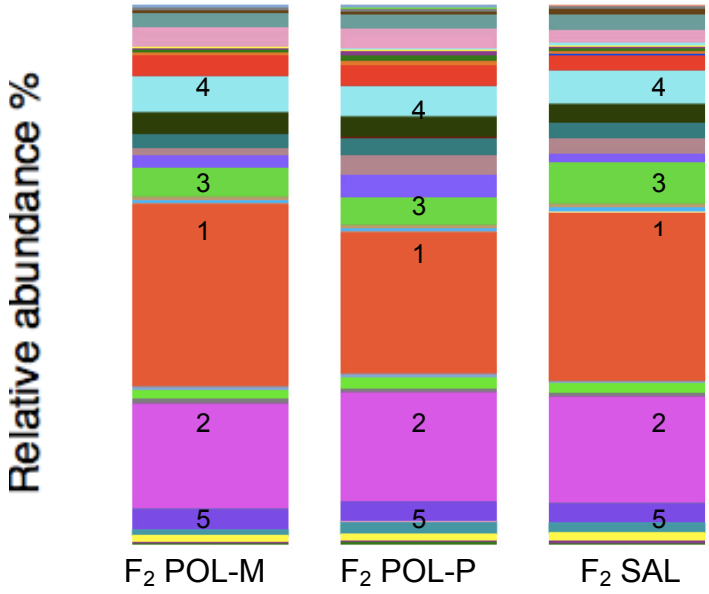


Figure 28. Taxonomy assignment for the F_2 generation (15,000 sequences/sample normalization value) in the three treatment groups (POL-P: poly(I:C) paternal lineage POL-M: poly(I:C) maternal lineage, SAL: control) Order of *Clostridiales* (1) with ~32% (SAL and POL-M) and 26.0% (POL-P), the *S24-7* family (2) with ~20% in all groups, the family of *Lachnospiraceae* (3) with 5.2% (POL-M), 4.9% (POL-P) and 7.5% (SAL), the *Oscillospira* genus (4) with 6.5% (POL-M), 5.6% (POL-P) and 5.8% (SAL) and the *Rikenellaceae* family (5) with 3.9% (POL-M) 3.6% (POL-P) and 3.6% (SAL).

6.3.2 Significant OTU selection. A different quantity of significant OTUs was obtained by analyzing the SAL group versus each one of the F_2 POL groups, 16 for F_2 POL-M lineage and 23 for F_2 POL-P lineage. The complete tables with all the significant OTUs could be visualized in Annex3.

6.3.2.1 POL-P lineage. Most of the significant OTUs found between F_2 POL-P and SAL groups belong to the *Firmicutes* and *Bacteroidetes* phylum and others belong to the *Tenericutes* and *Verrucomicrobia* phylum, as follows:

- Several genus of the order *Clostridiales,* the *Mogibacteriaceae, Lachnospiraceae* and *Ruminococcaceae* families, and the *Ruminococcus* and *Lactobacillus* genus from the *Clostridiales* order.
- Four *S24-7* families from the *Bacteroidales* order.
- The order *RF39* and the *Anaeroplasmatales* order from the *Tenericutes* phylum.
- The specie *Muciniphila* from the *Verrucomicrobiales* order.

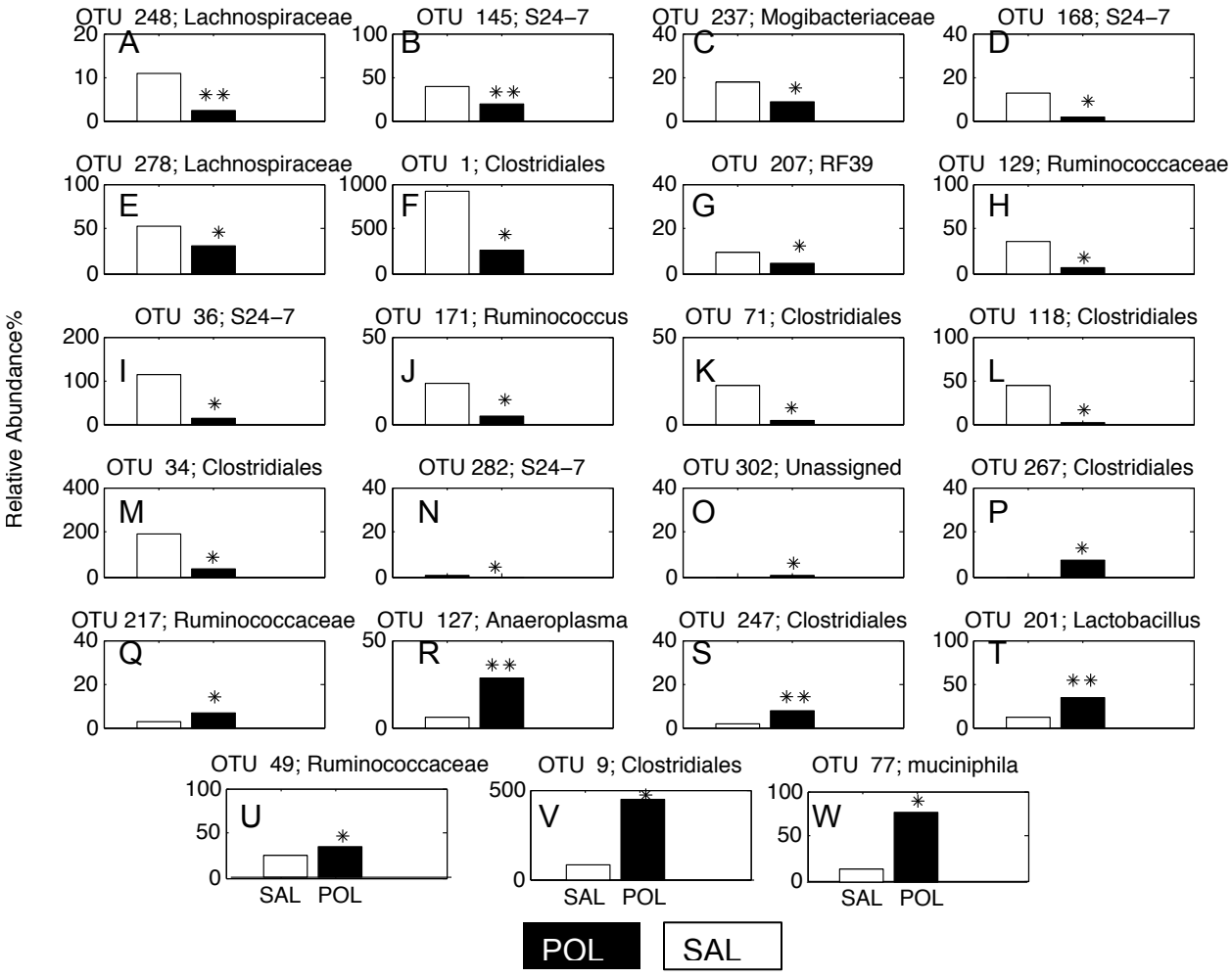


Figure 29. Significant OTUs present between treatment groups (POL: poly(I:C) and SAL: control) in the F2 generation POL-P lineage.
The first 14 OTUs A) Lachnospiraceae, B) S24-7, C) Mogibacteriaceae, D) S24-7, E) Lachnospiraceae, F) Clostridiales, G) RF39, H) Ruminococcaceae, I) S24-7, J) Ruminococcus, K) Clostridiales, L) Clostridiales, M) Clostridiales, and N) S24-7, are less abundant in the POL group, the last 9 OTUs O) Unassigned, P) Clostridiales, Q) Ruminococcaceae, R) Anaeroplasma, S) Clostridiales, T) Lactobacillus, U) Ruminococcaceae, V) Clostridiales and W) Muciniphila are more abundant in the POL group.

The OTUs: 248 *Lachnospiraceae* (Fig 29-A)), 145 *S24-7* (Fig 29-B), 168 *S24-7* (Fig 29-D), 237 *Mogibacteriaceae* (Fig 29-C), 278 *Lachnospiraceae* (Fig 29-E), 1 *Clostridiales* (Fig 29-F), 207 *RF39* (Fig 29-G), 129 *Ruminococcaceae* (Fig 29-H), 36 *S24-7* (Fig 29-I), 171 *Ruminococcus* (Fig 29-J), 71 *Clostridiales* (Fig 29-K), 34 *Clostridiales* (Fig 28-L), 282 *S24-7* (Fig 29-N) and 118 *Clostridiales* (Fig 29-O) showed less significant abundance in the POL-P group with respect the SAL group. The OTUs: 127 *Anaeroplasma* (Fig 29-R), 247 *Clostridiales* (Fig 29-S), 201 *Lactobacillus* (Fig 29-T), 49 *Ruminococcaceae* (Fig 29-U), 9 *Clostridiales* (Fig 29-V), 77 *Muciniphila* (Fig 29-W), 302 *Unassigned* (Fig 29-O), 267 *Clostridiales* (Fig 29-P) and 217 *Ruminococcaceae* (Fig 29-Q) showed more significant abundance in the POL-P group with respect the SAL group (Fig 29 A-M).

6.3.2.2 POL-M lineage. Most of the significant OTUs found between maternal lineage F_2 POL-M and SAL groups belong to the *Firmicutes* and *Bacteroidetes* phylum and other belong to the *Actinobacteria* phylum:

- Several orders of *Clostridiales,* the *Lachnospiraceae* and *Ruminococcaceae* families, and the *Oscillospira* genus from the *Firmicutes* phylum.
- *S24-7* and *Rikenellaceae* families from the *Bacteroidetes* phylum.
- The *Adlercreutzia* genus from the *Actinobacterias* phylum.

The OTUs: 203 *Rikenellaceae* (Fig 31-A), 59 *Oscillospira* (Fig 31-B), 63 *Clostridiales* (Fig 31-C), 118 *Rikenellaceae* (Fig 31-D), 84 *Oscillospira* (Fig 31-E), 25 *Clostridiales* (Fig 31-F), 174 *S24-7* (Fig 31-G), 82 *Lachnospiraceae* (Fig 31-H) and 168 *Oscillospira*

(Fig 31-I), showed less significant abundance in the F_2 POL-M group with respect the F_2 SAL.

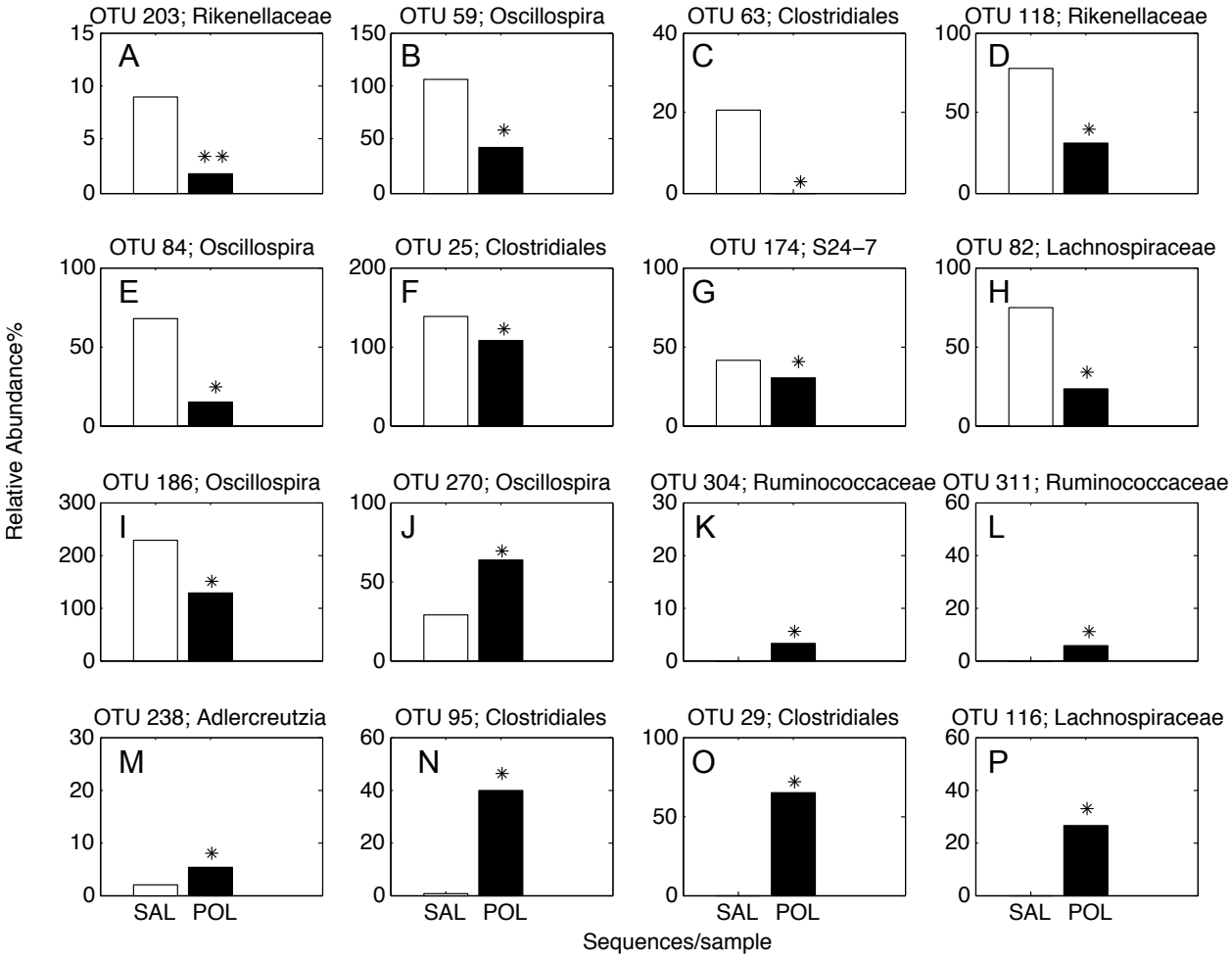


Figure 30. Significant OTUs present between treatment groups (POL: poly(I:C) and SAL: control) in the F_2 generation POL-M lineage.
The first 9 OTUs: A) Rikenellaceae, B) Oscillospira, C) Clostridiales, D) Rikenellaceae, E) Oscillospira, F) Clostridiales, G) S24-7, H) Lachnospiraceae and I) Oscillospira less abundant in the POL group, the last 7 OTUs: J) Oscillospira, K) Ruminococcaceae, L) Ruminococcaceae, M) Adlercreutzia, N) Clostridiales, O) Clostridiales, P) Lachnospiraceae are more abundant in the POL group.

The OTUs: 95 *Clostridiales* (Fig 31-N), 29 *Clostridiales* (Fig 31-O), 116 *Lachnospiraceae* (Fig 31-P), 270 *Oscillospira* (Fig 31-J), 304 *Ruminococcaceae* (Fig 31-K), 311 *Ruminococcaceae* (Fig 31-L) and 238 *Adlercreutzia* (Fig 31-M) showed

more significant abundance in the F_2 POL-M group with respect the F_2 SAL group (Fig 30 A-I).

6.3.4 Phylogenetic analysis. The Figure 31 shows a set of *Barnesiella* species OTUs: 42, 69 and 400 F_1 generation, 36, 145, 168 and 282 F_2 POL-P, and 174 F_2 POL-M, from the *Bacteroidetes* phylum, showed a direct transmission from the F_1 to the F_2 generation (as already reported in the second objective) specially to the F_2 generated by a POL-P. When we separated by lineages it can be observed that the association of the *Barnesiella* OTUs followed a direction in abundance (which couldn't be found in the second objective), all of them showed less abundance presence in the POL groups with respect the SAL groups in both F_1 and F_2 generations. Include that 4 of the 5 F_2 OTUs were generated by a POL-P and only one by a POL-M.

Therefore we have evidence that document that the paternal lineage is preponderant in the shaping of bacterial communities in the gut microbiota of poly(I:C) F_2. Interestingly linked with the results of the transgenerational effects of prenatal infection[17], were their data further suggested that the transgenerational effects in behavior of prenatal immune activation occur also via the paternal lineage. The authors tempted to speculate that prenatal immune activation may induce transgenerational effects via epigenetic modifications in male gametes, which might also affect gut microbiota composition indirectly.

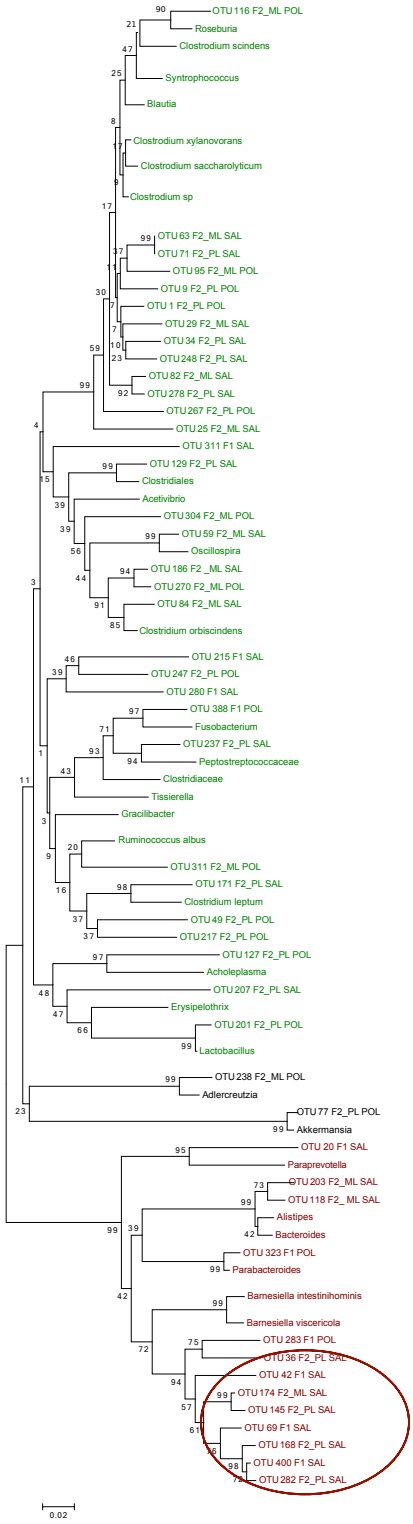


Figure 31 . Phylogenetic tree of the significant OTUs transmitted from the first F1 to the F_2 generation taking into account the paternal (POL-P) or maternal (POL-M) lineage. (The numbers in each branch are the values of the Bootstrap and the length of the branches the phylogenetic distance) **Firmicutes phylum: Green** / **Bacteroidetes phylum: red** OTUs *Barnesiella* are transmitted from to first to the F_2 generation and show a less abundance presence in POL groups with respect the SAL in F_1, and in the F_2 most of them in POL-P and one of them in POL-M.

7. Conclusions

We proposed that: If the gut microbiota is a relevant factor on the etiology of schizophrenia, a microbial dysbiosis will be observed in the mouse model of prenatal infection, which might work as a microbial vector that might be also horizontally transmitted across generations. Therefore, we demonstrated the presence of a microbial dysbiosis in the gut microbiota of the F_1 and F_2 offspring and its transmission from one generation to the other.

For our first objective, we demonstrate that there were differences in the presence and abundance of bacterial communities in the gut microbiota between the F_1 generation immune challenged offspring with poly(I:C) and the control offspring, with specific bacterial communities of the orders *Clostridiales* and *Bacteroidales*. Differences in the assignment of specific significant OTUs families or genus were found between platforms (IM or IT), as well as the alpha diversity; however, both of them reported the irregularity in the gut microbiota considered as a dysbiosis in the F_1 generation.

For the second objective, our data of the F_1 generation agree with the previously reported by Hsiao[35], where they also indicated that changes in the diversity of the gut microbiota are specifically in *Clostridia* and *Bacteroidia* OTUs, they found as significant the bacterial communities of the families: *Lachnospiraceae, Ruminococcaceae, Erysipelotrichaceae, Alcaligenaceae, Byphyromonadaceae, Prevotellaceae, Rikenellaceae* and *un-classified Bacteroidales,* suggesting that specific *Lachnospiraceae*, along with other *Bacteroidial* species, may play a role in ASD pathogenesis, while other taxa may be protective. We found as significant changes on

other bacterial communities: *Mogibacteriaceae, Bacteroidales S24-7, Clostridiales, Prevotella* and *Distasonis* suggesting that *Mogibacteriaceae* and *Distasonis* may play a role in ASD pathogenesis, while *Prevotella* and another *Clostridiales* may be protective against the pathogenesis.

The F_2 generation also showed differences in the gut microbiota of immune challenged offspring with the orders *Clostridiales* and *Bacteroidales* as the primary drivers of the dysbiosis. We found that some of the significant OTUs were transmitted from the F_1 to the F_2 generation, more specifically some *Gracillibacter* species from order *Clostridiales* that presented less abundance in the POL group of both generations and *Barnesiella* species from the order *Bacteroidales* which don't clearly show a relationship between the transmission of these significant OTUs across generations and their abundance in the POL group vs. SAL group.

However, for the third objective were we separated the POL offspring according to the lineage for which they were generated, we demonstrated that the order *Bacteroidales* showed a less abundance presence in the POL groups with respect the SAL groups and were transmitted from the F_1 to a F_2 offspring mainly generated by a paternal lineage.

All our results have a direct relationship with the ones already obtained (transgenerational effects of prenatal infection[17]) in which our research is based. They demonstrated that prenatal viral-like immune activation leads to a transmission of behavioral phenotypes across generations, showing that reduced sociability and increased cued fear expression were similarly present in the first and second-generation

offspring of immune-challenged ancestors, that sensorimotor gating impairments were confined to the direct descendants of infected mothers, whereas increased behavioral despair emerges as a novel phenotype in the second generation, and that the effects were mediated via the paternal lineage, demonstrating transgenerational non-genetic inheritance of pathological traits following in-utero immune activation. For the F_1 generation, a behavior despaired in the POL group was presented with an impaired sociability, increased fear expression and a robust reduction in sensorimotor gating. Similarly, we found 10 different significant OTUs mainly of the orders *Clostridiales* and *Bacteroidales* that differed in abundance and presence of in the POL group in contrast to the SAL group. Furthermore, for the F_2 generation the responses were increased inasmuch as there was a rise in the behavioral despair, showing a similar deficit in sociability than F_1, an increased fear expression, and by contrast with the F_1 offspring that present an absence of behavioral despair associated with depressive-like behaviors, the F_2 offspring developed signs of behavioral despair, we obtained a greater number of significant OTUs also from the orders *Clostridiales* and *Bacteroidales*, that present a different abundance in the of POL and SAL groups creating the dysbiosis in the gut microbiota of the immune challenged offspring.

In conclusion, we demonstrated that the gut microbiota is a relevant factor on the etiology of schizophrenia and ASD, inasmuch as a microbial dysbiosis was observed in the mouse model of prenatal infection, and was horizontally transmitted across generations and that the paternal lineage is a preponderant linage for the transmission of bacterial communities from the F_1 to the F_2 generation. Which positions the gut microbiota as a possible vector of transgenerational transfer. However, it is important to

mention that our research had some limitations, first of all, we didn't analyze the gut microbiota composition of the 'generation zero' F_0, given that we assumed that the gut microbiota would be equal or equivalent to the control F_1. However, our own data indicated that the microbiota of the control groups varies between generations (F_1 vs. F_2). Additionally, our experimental design remains at a phenomenological level without any intervention to look for causality since it remains at a descriptive status. So that, for further research a new experimental design should be raised that includes and takes into account these limitations, specially to look for the causality of the already obtained results.

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- Ma. en C. Gabriela Bravo: extracción y amplificación de DNA de muestras para Ion Torrent.
- Dr. Félix Aguirre: apoyo en el análisis y procesamiento bioinformático de los datos

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