**Routine use of probiotics in preterm infants: longitudinal impact on the microbiome and metabolome**

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Short title - probiotics in preterm infants

Keywords – Preterm, gut microbiota, probiotics, NGS, metabolomics

Word count - 2449

**ABSTRACT**

**Objective**

Probiotics are live microbial supplements that colonize the gut and potentially exert health benefit to the host. We aimed to determine the impact of probiotic (Infloran®: *Lactobacillus acidophilus*-NCIMB701748 and *Bifidobacterium bifidum*-ATCC15696) on the bacterial and metabolic function of the preterm gut while on the neonatal intensive care unit (NICU) and following discharge.

**Patients**

Stool samples (*n = 88*) were collected before, during, and after probiotic intake from 7 patients, along with time-matched controls from 3 patients. Samples were also collected following discharge home from the NICU.

**Methods**

Samples underwent bacterial profiling analysis by 16S rRNA gene sequencing and quantitative PCR (qPCR), as well as metabolomics profiling using liquid chromatography mass spectrometry (LCMS).

**Results**

Bacterial profiling showed greater *Bifidobacterium* (15.1%) *and Lactobacillus* (4.2%) during supplementation compared to the control group (4.0% and 0%, respectively). While *Lactobacillus* reduced after probiotic was stopped, *Bifidobacterium* remained high following discharge, suggestive of successful colonisation. qPCR analysis showed a significant increase (*P =* <0.01) of *B. bifidum* in infants who received probiotic treatment compared to controls, but no significant increase was observed for *L. acidophilus* (*P =* 0.153). Metabolite profiling showed clustering based on receiving probiotic or matched controls, with distinct metabolites associated with probiotic administration.

**Conclusions**

Probiotic species successfully colonise the preterm gut, reducing the relative abundance of potentially pathogenic bacteria, and effecting gut functioning. *Bifidobacterium* (but not *Lactobacillus*) colonized the gut long-term; suggesting the possibility that therapeutically administered probiotics may continue to exert important functional effects on gut microbial communities in early infancy.

**INTRODUCTION**

Probiotics are live microorganism which when administered in an appropriate amount will confer a health benefits to the host.[1] Globally *Lactobacillus* spp*.* and *Bifidobacterium spp.* are most commonly used.[2] For preterm infants, probiotic use has focused on potential reduction in necrotising enterocolitis (NEC) and late onset sepsis (LOS),[3] but other important outcomes (feed tolerance, asthma, allergy) may be relevant.[4] Evidence from more than 20 RCTs suggests probiotics reduce NEC.[5] Meta-analysis shows a relative risk (RR) of 0.43 (95% CI 0.33-0.56) for NEC, and no impact on LOS (RR 0.91 95% CI 0.8-1.03). [6,7]

Mechanisms of probiotic action in preterm are multiple and complex and include potential competition with pathogenic bacteria in the gut ecosystem, direct effects on naive host immune mechanisms and enhancing intestinal maturation,[8] including enhancing tight junction integrity.[2] Data relating to probiotic mechanisms *in vivo* in preterm infants are limited: few trials report colonisation rates or peristence, or factors affecting these. Two ongoing/recently completed studies included stool microbiology analysis (Cooper NCT00977912 and Costeloe ISRCTN05511098) but are not yet published. However, dysbiosis preceding NEC has been well described.[8–12]

In order to better understand the potential role of probiotics we studied preterm infants with molecular sequencing and metabolomic techniques. Metabolomics is a relatively new technique to determine functional impacts of changes in gut microbial communities by analysing stool metabolites. When combined with microbiome analysis, in well phenotyped cases, this can improve our mechanistic understanding of health and disease and the effects of clinical interventions like probiotics. We aimed to conduct a longitudinal examination of extremely preterm infants through a neonatal intensive care unit (NICU) and post discharge to determine the effects of therapeutic exposure to the probiotic Infloran**®** (*B.bifidum*-ATCC-1569-6 and *L.acidophilus*-NCIMB 701748). We explored changes in the overall bacterial community (16S rRNA profiling) and accurately quantified the probiotic species (qPCR). In addition, ultra-performance liquid chromatography mass spectrometry tandem mass-spectrometry (UPLC-MS/MS) stool metabolomic profiling was undertaken to ascertain if probiotic administration resulted in functional shifts at the metabolite level.

**METHODS**

**Study design**

Ethics approval was obtained from County Durham and Tees Valley Research Ethics Committee, and parents gave signed consent for stool and data collection.

Infloran was introduced in January 2013 with the aim of giving it to all infants <32 weeks gestation soon after initial introduction of feeds until 34 weeks corrected. All were born within 3 months: we also identified a small number of infants who met the criteria but did not receive probiotics. This contemporaneous group provided us with an opportunity to explore cross-colonisation of probiotic strains in our NICU. Overall, 88 stools were analysed from 10 patients (7 probiotic and 3 controls). Of these, 60 were from probiotic exposed infants (10 before, 32 during, 14 after exposure and 4 after discharge) and 28 were from as day of life (DOL) matched control infants. All samples underwent 16S bacterial profiling and of these a representative subset underwent quantitative PCR (qPCR) (*n =* 75; 50 probiotic (5 before, 30 during, 11 after and 4 PD) and 25 control). All samples where sufficient stool remained also underwent metabolomics profiling (*n* = 40; 28 probiotic (1 before, 17 during, 6 after and 4 PD) and 12 control). This number of samples compares favourably with previous work and allows gut profiles to be confidently discriminated.[12,13]

All patients received some maternal breast milk additional demographics and sampling in relation to probiotic exposure and age are summarised in Table 1.

**Table 1 - Demographic data for the clinical cohort**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | Control | | | Probiotic | | | | | | |
|
| Patient ID | 263 | 271 | 272 | 270 | 273 | 274 | 275 | 276 | 277 | 278 |
| Gestational age (weeks) | 27 | 31 | 31 | 25 | 27 | 24 | 28 | 28 | 24 | 24 |
| Birth weight (g) | 550 | 2030 | 1535 | 750 | 945 | 700 | 1100 | 1150 | 620 | 620 |
| Delivery mode | CS | V | V | V | CS | V | CS | CS | CS | CS |
| Pathology | NA | NA | NA | Surgical NEC  day 4 | NA | S. epidermidisLOS  day 14 | NA | NA | Medical NEC  day 28 + CoNS LOS day 13 | NA |
| Live | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | No | Yes |
| Sex | F | F | M | M | F | M | F | M | F | M |
| DOL on probiotics | - | - | - | 31-61 | 14-28 | 12-70 | 3-12 | 8-26 | 14-26 | 5-73 |
| Samples  N, (DOL Range, median) | 10 (12-108, 55) | 7 (3-35, 14) | 7 (3-35, 14) | 7 (9-77, 59) | 7 (9-54, 40) | 12 (9-64, 29) | 5 (6-21, 10) | 4 (9-29,19) | 3 (6-27,16) | 8 (7-38, 17) |
| Post discharge age (weeks) | NA | NA | NA | 62 | 60 | 59 | NA | NA | NA | 54 |
| Antibiotics course combination | 0(P2, g2) 6 (M2,C2,V2) 30 (A6,F6,G6) | 0(P2, G2) | 0(P2, G2) | 0(P,G2) 3 (C5,V7,M8) 14 (C5,V5,M5) 28 (C2,V2) 33 (C2,V2) | 0 (P2, G2) | 0(p,2 g2), 14 (V2, m2, c2) 36 (v4,c4,m4) 51 (v2,c2) y | 0(P2, G2) | 0(P2, G2) | 0(P2G2) 12(V7,C7,M7) | 0(P2G2) 11 (A3,F3,G3) 23(A2,F2,G2) 28 (A2,F2,G2) 59 (A2,G2,F2) |

**Analysis of Faecal microbiota composition by 16S bacterial profiling**

Bacterial DNA was extracted from 100 mg of stool using PowerLyzer™ PowerSoil® DNA Isolation Kit (MoBio) according to manufacturer’s instructions. Bacterial was carried out by NU-OMICS (Northumbria University) based on the Schloss wet-lab MiSeq SOP (available at - <http://www.mothur.org/wiki/MiSeq_SOP>). Raw fastq data were processed using Mothur (version 1.31.2) as described in the MiSeq SOP.[14] Sequences were deposited in MG-RAST under the accession numbers 4615668.3-4615749.3.

**Analysis of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* by qPCR**

To generate standard curves, PCR products (Table 2) were cleaned using Exo-SAP-IT and cloned into pGEM-T easy vector (Promega) and expressed in *E. coli* K12 cells.[15,16] Plasmid DNA was extracted using the PureYield™ PlasmidMiniprep kit (Promega), quantified using NanoDrop 1000 (Thermo Scientific) and used to generate standards from X-Y copies/g. Absolute quantification was carried out in a CFX96 (Bio-Rad) using ABsolute QPCR SYBR Green mix (Thermo Scientific).

**Table 2: Primers used in qPCR**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Species | Name of  Primer | Sequence (5'-3') | Target  sequence | Annealing temperature (°C) | Product  Size (bp) | Reference |
| *B. bifidum* | BiBIf- 1 | CCACATGATCGCATGTGATTG | 16S rDNA | 62 | 278 | Matsuki *et al*., 2004 |
| BiBIF- 2 | CCGAAGGCTTGCTCCCAAA |
| *L. acidophilus* | Acidfor | AGCGAGCTGAACCAACAGAT | 16S rDNA | 60 | 227 | Tabasco *et al*., 2007 |
| Acidrev | AGGCCGTTACCCTACCAACT |

**Metabolomic profiling**

Water, methanol, and acetonitrile (ACN) were LCMS grade (Sigma-Aldrich). Metabolites were extracted from 100mg stool and homogenised in cold 80% methanol by vortexing for 15 min at 4oC. The suspension was then centrifuged at 10,000×g for 10 min at 4oC and lyophilised. Samples were re-suspended in 1 ml and diluted a further 1:1 in initial start phase buffer (5% ACN).

Stool metabolites profiling was performed using reverse-phase UPLC-MS/MS. An Accucore C18 column (2.6 µm, 150 × 2.1 mm) was used at 40 ºC with a 3.0 µl injection and 300 µl/min flow rate throughout. A multi-step LC gradient was used with 5% ACN increasing to 95% ACN over 22 minutes, with a further 95% ACN for 4 minutes followed by a final 4 minutes re-equilibration at 5% ACN. Samples were run in triplicate and the order in each triplicate sequence randomised. A Q-Exactive (Thermo) was used for the MS and subsequent data dependant MS/MS. Metabolomic profiling was performed using HESI with high resolution (70,000) positive and negative switching. The mass range was set from 100 – 1,000 m/z.

SIEVE (Version 2.2 *beta*) was used to process the Thermo RAW files by component extraction. All blanks were used in background subtraction. Positive and negative data were processed individually and combined prior to downstream analysis.

**Statistical analysis**

The bacterial profiles were analysed by multivariate partial least squares discriminant analysis (PLS-DA) and the metabolite profiles underwent orthogonal PLS-DA (OPLS-DA) using SIMCA 13.0 (Umetrics, Stockholm, Sweden).[17] All variables, either operational taxonomic unit (OTU) or component, were automatically transformed within SIEVE. To check that data was adhering to multivariate normalities, Hotelling’s *T2* tolerance limits were calculated and set at 0.95. To remove the high amounts of noise from the metabolomics dataset, only variables >1 in the variable importance plot (VIP) were included from the important variables plot.[18] Analysis of variance (ANOVA) was used to determine whether numbers of *L. acidophilus* and *B. bifidum* (qPCR) increased during probiotic treatment with post-hoc Tukey’s applied for multiple pairwise comparisons (Minitab 16).

**RESULTS**

**16S bacterial profiling of the gut microbiota**

Infants exposed to Infloran® had distinct gut microbiota profiles compared to controls (Figure 1A). The control group cluster independently irrespective of time points, the probiotic group cluster together before, during and after exposure. Using >97% similarity cut-off applied in determining OTUs, three different OTUs identified as *Lactobacillus* and one *Bifidobacterium* OTU were associated with current probiotic exposure (Figure 1B). Most of the *Lactobacillus* spp. had the highest abundance during probiotic treatment, but these OTUs persisted after probiotic treatment had stopped. No *Lactobacillus* OTUs associated with probiotic administration were detected in controls and one *Lactobacillus* OTU was present at 0.1% relative abundance in the PD samples (Table 3 and Supplementary Figure 1). In contrast, the abundance of *Bifidobacterium* was relatively high before probiotic supplementation (11.1%) and in controls (4.0 %), but the abundance of this OTU continued to increase during (15.0%) and following (19.1%) probiotic administration in treated infants, falling slightly PD (14.2%). While the gut microbiota was distinct *between* patients receiving probiotics and controls (R2Y = 0.85, Q2 = 0.60), the gut microbiota *within* patients remained relatively comparable while receiving probiotics (R2Y = 0.36, Q2 = 0.27), compared to before (R2Y = 0.18, Q2 = 0.02), and after (R2Y = 0.25, Q2 = 0.09). Here R2Y represents how well the variable is explained and Q2 represents how well the variable is predicted. Thus demonstrating addition of probiotic OTUs, rather than large shifts in the overall gut microbiota as a result of probiotic administration.

The OTUs associated with control samples included a *Clostridium* (3.84% in controls compared with 0.47% in probiotic, *P* = 0.002) and a *Streptococcus* (3.09% in controls compared with 0.01% in probiotic, *P* = 0.001). OTUs present in relatively high abundance in PD samples include *Anaerostipes* (4.37%), *Bacteroides* (10.36%), *Lachnospiracea* (5.1%), *Proteus* (5.53%), and *Veillonella* (4.47%).

The Shannon diversity of the control group was higher than the probiotic group, with the during probiotic samples being significantly lower (*P* = <0.001) (Figure 2). Within the probiotic groups the Shannon diversity increased across time. All groups had a significantly lower (*P* = <0.001) diversity compared to the PD samples.

**Table 3 – Relative percentage abundance of select OTUs**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Control | NICU Probiotic | | | PD |
| Before | During | After |
| *Acinetobacter* | **1.38** | 0.02 | 0.00 | 0.00 | 0.00 |
| *Actinomyces* | **2.36** | 0.00 | 0.00 | 0.00 | 0.00 |
| *Anaerostipes* | 0.00 | 0.00 | 0.00 | 0.00 | **4.37** |
| *Bacteroides* | 0.00 | 0.00 | 0.03 | 0.00 | **10.36** |
| *Bifidobacterium* | 4.02 | **11.06** | **15.03** | **19.10** | **14.16** |
| *Clostridium1* | **3.84** | 0.03 | 0.02 | 0.47 | 0.00 |
| *Clostridium2* | **0.81** | 0.00 | 0.00 | 0.00 | 0.08 |
| *Enterococcus* | **18.41** | **18.93** | **24.38** | 12.04 | 1.16 |
| *Escherichia* | 11.44 | 2.06 | 19.66 | **23.77** | 14.02 |
| *Klebsiella* | **37.41** | 7.93 | 16.54 | 23.92 | 12.84 |
| *Lachnospiracea* | 0.00 | 0.00 | 0.00 | 0.00 | **5.10** |
| *Lactobacillus1* | 0.00 | 1.73 | **4.81** | **4.77** | 0.00 |
| *Lactobacillus2* | 0.00 | 0.77 | **5.80** | 0.08 | 0.00 |
| *Lactobacillus3* | 0.00 | 0.73 | 0.20 | **2.32** | 0.01 |
| *Lactobacillus4* | 0.00 | 0.06 | **0.19** | **0.24** | 0.00 |
| *Proteus* | 0.00 | 0.00 | 0.00 | 0.00 | **5.53** |
| *Pseudomonas* | **1.58** | **1.44** | 0.49 | 0.03 | 0.01 |
| *Staphylococcus* | 5.86 | **53.49** | 9.73 | 10.52 | 0.02 |
| *Streptococcus1* | **3.09** | 0.01 | 0.01 | 0.00 | 1.04 |
| *Streptococcus2* | **1.00** | 0.00 | 0.00 | 0.00 | 0.00 |
| *Veillonella* | 0.00 | 0.00 | 0.00 | 0.00 | **4.47** |

**Quantification of *B.bifidum*-ATCC-1569-6 and *L.acidophilus*-NCIMB 701748 by qPCR**

In accordance with the OTU data, the *B. bifidum* load, (i.e copy number of this species), was significantly (*P =* <0.001) higher at all-time points following administration of probiotic (Figure 3A). *B. bifidum* was only detected in one probiotic infant before administration and was not detected in any control infants ever. *L. acidophilus*, the reported strain in Infloran, was only detected in quantifiable levels in 2 samples during probiotic administration and in 3 control samples (Figure 3B).

**Metabolomics profiling of the gut microbiota**

Overall, metabolite profiles demonstrated comparably more uniformity than bacterial profiles. The PD samples were found to have a distinct metabolite profile when compared with NICU samples by OPLS-DA (R2Y = 0.99, Q2 = 0.8), with a strong association of unique metabolites and metabolites present in much greater abundance compared with NICU samples (Supplementary Figure 2). Due to low amount of sample in the ‘before’ samples (and the skew caused by the PD samples), these 2 groups were omitted from OPLS-DA of the probiotic and control groups. Due to the inherently similar metabolomics profiles, OPLS-DA was unable to robustly separate samples taken during or after probiotic administration, and control groups (Figure 4A: R2Y = 0.62, Q2 = 0.31). However, following removal of metabolites with a VIP of <1 (leaving only significant metabolites with large differences in the relative intensities), the groups could be separated more robustly (Figure 4B: R2Y = 0.80, Q2 = 0.56). The loadings plot showed a cluster of metabolites associated with samples during probiotic intake. Further study will be required to robustly identify these and other metabolites of interest using additional techniques or known standards.

**DISCUSSION**

We explored the gut microbiota in preterm neonates receiving the probiotic Infloran and non-exposed contemporaneous controls. We also explored the persistence of effects after discharge of receiving probiotics on the NICU. Uniquely, both molecular sequencing and metabolomic profiling was performed to determine, 1) overall changes to the gut microbiota as a result of probiotic receipt, 2) the quantification of the reported species in Infloran, 3) long-term colonisation, and 4) associated functional shifts in gut metabolites.

Supervised modelling demonstrated that infants receiving Infloran have a different gut microbiota to controls, where the probiotic genera used in this study (*Bifidobacterium* and *Lactobacillus*) were most associated with samples taken during probiotic receipt. During on-going administration, such detection is not necessarily indicative of colonisation but may simply represent the passage of DNA from the administered species through the host.[19] However, by exploring persistence following administration ceased and after discharge we found the *Bifidobacterium* OTU associated with probiotic intake was able to successfully establish in the resident flora long-term, with comparable levels PD as during active administration. Although we attempted to obtain PD control samples none were obtained, preventing direct comparison.

*Bifidobacterium* OTUs were present in control samples and before probiotic administration in the NGS data, but the strain of *B. bifidum* used in the probiotic was not detected by qPCR in controls and was detected in only 1 ‘before’ probiotic sample. While the NGS and qPCR data were in agreement for *B. bifidum*, data were less comparable for *L. acidophilus*. By NGS *Lactobacillus* OTUs were not detected from controls, and scarcely detected before probiotics or PD. However, three *Lactobacillus* OTUs increased during and after probiotic, compared with control and PD samples. Multiple OTUs of *Lactobacillus* may represent issues in the bioinformatic alignment and identification, or the potential that more than one species of *Lactobacillus* was present in Infloran. The enhanced ability of *Bifidobacterium* to successfully colonise and proliferate with higher abundance and greater persistence compared to *Lactobacillus* is important, and may suggest the potential differing probiotic efficacy in clinical trials. Increased colonisation by *Bifidobacterium* over *Lactobacillus* has previously been shown in one breastfed preterm population,[20] but opposing trends with increased colonisation by *Lactobacillus* have been shown for term neonates.[21]

Administration of probiotic did not significantly alter the total composition of gut microbiota, with most bacterial OTUs being detected in all samples. However, changes in the relative *abundance* of OTUs did occur in probiotic infants, with an increased abundance of *Bifidobacterium* and *Lactobacillus* and notable decreases in the relative abundances of other taxa. The relatively high abundance of *Staphylococcus* spp. before probiotics were administered likely reflects high rates of caesarean delivery in probiotic infants.[22,23] Organisms detected almost exclusively in controls included *Acinetobacter, Actinomyces, Clostridium,* and *Streptococcus,* agreeing with published data.[20,24] Importantly, a significant reduction in organisms associated with NEC or LOS, including *Clostridium* and *Klebsiella*, were found in probiotic infants.[25,26]

In accordance with a small number of published studies, the metabolomic profiles were more comparable and stable than bacterial profiles.[11,27] although the metabolomics profiles of probiotic infants were comparable to those of controls, certain metabolites were detected with significantly increased abundance during probiotic administration. None of these metabolites could be matched with our existing internal standards and further work will be needed to identify these, determining whether they may be connected with potential regulatory pathways.

The data presented are from a small number of infants contributing variable numbers of samples at different postnatal ages, but are well representative of before, during and after probiotic administration. With the potential for probiotic strains to cross-contaminate other infants within the NICU environment, it is important that all infants were resident contemporaneously. Despite the sampling limitations of our study, significant cross-colonisation in our unit using this product seems un-common.

In summary, probiotic species alter gut microbial communities during and after therapeutic administration, with *Bifidobacterium* but not *Lactobacillus* demonstrating long-term proliferation in this population. A reduction in the abundance of taxa such as Clostridia that have been associated with NEC may be relevant if, as the meta-analyses suggest, probiotics do reduce the occurrence of this serious disease. Multiple OTUs associated with *Lactobacillus* were detected, although qPCR was unable to robustly quantify the *L. acidophilus* used in the administered product, thus raising important quality assurance concerns exist around the use of commercially available products in a high-risk patient group. Exploring functional metabolite changes may provide important information on mechanisms of action of interventions such as probiotics, and should be considered for inclusion into future interventional trials.

**COMPETING INTERESTS**

None to declare.

**FUNDING**

This research was funded in part by Danone Nutricia. Funders played no part in the study design, in the collection, analysis and interpretation of data; in the writing of the manuscript; or in the decision to submit the manuscript for publication.

**CONTRIBUTORSHIP**

CJS, NDE, JEB, SPC and JDP contributed to the study idea and design. BA, CJS, AN, TS, JEB, ECLM, and NDE contributed to collection of samples and data. CJS, BA, AN, JEB, NDE, and SPC contributed to analysis of samples and data. All authors have contributed to and reviewed the manuscript.

**What is already known on this topic?**

* The preterm gut microbiota has important influences in the onset of necrotising enterocolitis (NEC) and late onset sepsis (LOS)
* Probiotics potentially reduce the risk of NEC and LOS

**What this study adds**

* *Bifidobacterium* but not *Lactobacillus* demonstrated long-term proliferation in this population
* Probiotic species colonise the gut and reduce the prevalence of potentially pathogenic organisms
* Colonisation by probiotic bacterial species causes metabolomics shifts in gut functioning

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**Figure legends**

**Figure 1** **- PLS-DA of 16S bacterial profiles**. Post discharge samples removed. A) Score scatter plot. B) Loadings plot

**Figure 2 – Box plot of Shannon Diversity Indices**. Significantly lower diversity occurred in during probiotic administration compared to controls and in all groups when compared to samples post discharge (PD).

**Figure 3 - Boxplot of bacterial load determined by qPCR.** \*Asterisks represent the outliers, “a” or “b” represents the grouping by turkey’s family. A)*Bifidobacterium bifidum*. B) *Lactobacillus acidophilus*.

**Figure 4 -** **PLS-DA of the metabolite profiles**. Before probiotic and post discharge samples excluded. A) Score scatter plot showing the relationship of samples associated with each group (R2Y = 0.62, Q2 = 0.31). B) Loading plot of detected metabolites associated with each group following VIP removal of noise (R2Y = 0.80, Q2 = 0.56). Red metabolites associated with probiotic administration.

**Supplementary Figures**

**Supplementary Figure 1** **- Relative abundance of bacterial OTUs**. Sequences matching other less abundant OTUs are not shown on the legend

**Supplementary Figure 2** **– OPLS-DA of metabolite profiles comparing samples collected on the NICU and post discharge**. R2Y = 0.99, Q2 = 0.8. A) Score scatter plot. B) Loadings plot.