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Gut microbiota-based prediction for the transition from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) in a remote island cohort study

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ABSTRACT

Aim: The present cohort study explored whether specific gut microbiota (GM) profile would predict the development of impaired glucose tolerance (IGT) in individuals with normal glucose tolerance (NGT).
Methods: A total of 114 study subjects with NGT in Kumejima island, Japan participated in the present study and underwent 75 g oral glucose tolerance tests at baseline and one year later. We compared the profile of GM at baseline between individuals who consistently maintained NGT (NRN, n = 108) and those who transitioned from NGT to IGT (NTI, n = 6).
Results: Within-individual bacterial richness and evenness as well as inter-individual bacterial composition showed no significant differences between NRN and NTI. Of note, however, partial least squares discriminant analyses revealed distinct compositions of GM between groups, with no overlap in their 95 % confidence interval ellipses. Multi-factor analyses at the genus level demonstrated that the proportions of *CF231, Corynebacterium, Succinivibrio,* and *Geobacillus* were significantly elevated in NTI compared to NRN ($p < 0.005$, FDR < 0.1, respectively) after adjusting for age, sex, HbA1c level, and BMI.
Conclusions: Our data suggest that increased proportion of specific GM is linked to the future deterioration of glucose tolerance, thereby serving as a promising predictive marker for type 2 diabetes mellitus.

1. Introduction

Impaired Glucose Tolerance (IGT) is identified as a prediabetic condition affecting over 540 million adults worldwide [1]. It has been reported that IGT is a borderline status with about 25 % of individuals developing diabetes mellitus (DM) within three to five years [2]. Importantly, even individuals with IGT show a considerably high incidence of cardiovascular events including coronary heart disease and stroke, and all-cause mortality compared to those with normal glucose tolerance (NGT) [3].

Recently, time course of aggravation in glucose tolerance has been shown to link with a loss of diversity and/or imbalance of gut microbiota, also known as dysbiosis [4], that may negatively affect fuel homeostasis in both humans and rodents [5]. For example, abundance of *Bacteroides* is negatively correlated with the host's insulin resistance [6]. On the other hand, that of *Faecalibacterium* species are associated with

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anti-inflammatory status in subjects with obesity or DM, independently of calorie intake [7]. Even in subjects with IGT, an apparent decrease of abundance already occurred in beneficial bacteria such as *Alistipes* [8] and *Akkermansia* [9], but conversely, an apparent increase was observed in opportunistic pathogens like Enterobacteriaceae. However, to the best of our knowledge, a detailed characterization of the gut microbiota that might predict the transition from NGT to IGT has not yet been fully conducted.

In this context, we focused our attention on Kumejima island in Okinawa, the southernmost part of Japan, which provides us with a unique opportunity for epidemiological studies on lifestyle-related diseases. Despite historical reputation for healthy longevity, the rapid adoption of Western diets and sedentary lifestyle has led to a rise in obesity and T2DM in Okinawa, particularly in remote islands [10]. Taken together, investigating the gut microbiota in Kumejima residents is of unique value to gain further insight into the prevention of lifestyle-related diseases. In fact, in the precedented study using the same cohort, we identified specific gut microbiota and serum metabolites capable of distinguishing between metabolically healthy obesity (MHO) and metabolically unhealthy obesity (MUO) [11]. Based on these backgrounds, the present study aimed to explore the potential of specific gut microbiota as a predictive marker for the risk of transitioning from NGT to IGT, thereby contributing to the unique proposal of preventive strategies against T2DM.

2. Methods

2.1. Study design and population

As a part of the Kumejima Digital Health Project (KDHP) between June 2018 and December 2019, this cohort study included residents in Kumejima island, Okinawa prefecture in Japan. KDHP is a kind of

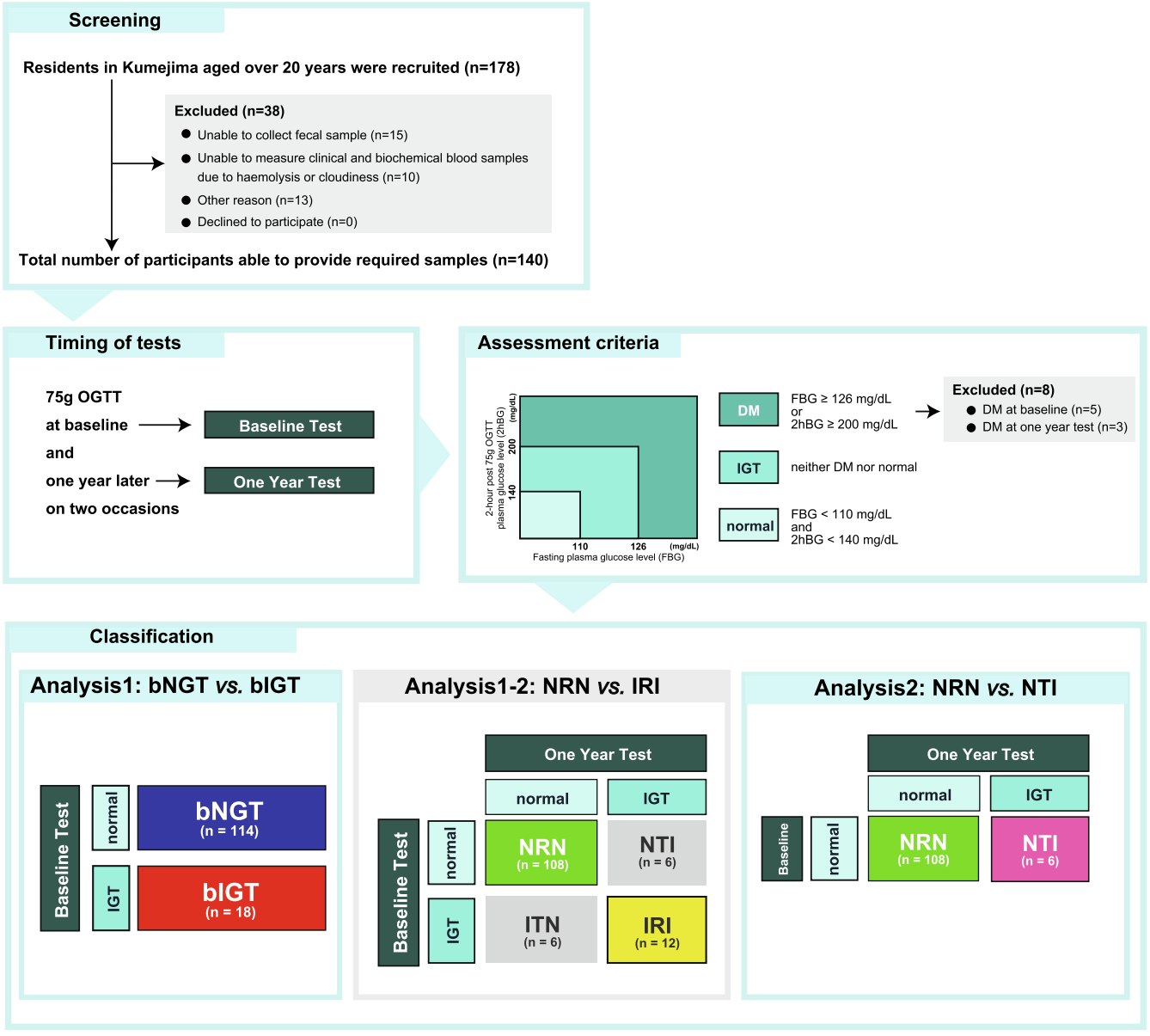

Fig. 1. Study scheme. bNGT; normal glucose tolerance at baseline (blue) (n = 114), bIGT; impaired glucose tolerance at baseline (red) (n = 18), NRN; consistent normal glucose tolerance at baseline and one year (green) (n = 108), NTI; normal glucose tolerance at baseline and impaired glucose tolerance at a year later (pink) (n = 6), ITN; impaired glucose tolerance at baseline and normal glucose tolerance at a year later (gray) (n = 6), IRI; consistent impaired glucose tolerance at baseline and one year later (yellow) (n = 12). 75 g OGTT; 75 g oral glucose tolerance test. Graphics were made using Adobe Illustrator 2024 (version: 28.3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Japanese national project designed to observe health status of residents in a remote island over time with a particular emphasis on the profile of gut microbiota. Subjects were residents of Kumejima island aged over 20 years, excluding those with the following conditions: 1) severe renal dysfunction (estimated glomerular filtration rate < 30 ml/min/1.73 m^2) or hepatic dysfunction (aspartate aminotransferase > 200 IU/L or alanine aminotransferase > 200 IU/L), 2) malignancy, 3) pregnancy, 4) chronic diarrhea, or 5) severe illness requiring hospitalization and treatment. A total of 178 subjects were recruited, among which 140 individuals were able to provide all the required measurements and samples including anthropometric measurements, clinical and biochemical blood samples as well as stool samples. Because we focused on IGT, 8 of the 140 subjects were excluded due to demonstrating diabetic patterns, defined as a fasting plasma glucose level of ≥ 126 mg/dL or a 120-minute plasma glucose level of ≥ 200 mg/dL during the 75 g OGTT [12], at baseline and one year later, based on the 75 g oral glucose tolerance tests (75 g OGTT). Therefore, 132 individuals (65 females and 67 males) without diabetic patterns at both time points were finally included for analyses **(**Fig. 1**, Upper)**.

All subjects provided written informed consent, and the present study was approved by the Ethics Committee of the University of the Ryukyus for Medical and Health Research Involving Human Participants (No. 1194) following the Declaration of Helsinki.

2.2. Sample and basic data collection

Venous blood samples were collected by cubital venipuncture after an overnight fast. Serum total cholesterol and triglyceride were measured by Determiner C-TC (Minaris Medical Co., Ltd., Tokyo, Japan), while serum high-density lipoprotein-cholesterol (HDL-cholesterol) was measured by Metabolead HDL-C (Minaris Medical Co., Ltd., Tokyo, Japan). Serum low-density lipoprotein-cholesterol (LDL-cholesterol) was calculated using the Friedewald equation: total cholesterol level – HDL-cholesterol level – (triglycerides level/5) [13]. Hemoglobin A1c (HbA1c) in blood was analyzed with Tosoh Automated Glycohemoglobin Analyzer HLC-723G8 (TOSOH Corporation, Shiga, Japan). Plasma glucose was measured by l-type Wako Glu2 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). Plasma immunoreactive glucagon (IRG) was measured by Glucagon RIA kit (SML) (DIAsource ImmunoAssays S.A., Brabant Wallon, Belgium). Blood pressure and anthropometric indices were measured by trained nurses in a standard fashion. Within 0–7 days of blood collection, subjects provided fecal samples, using a fecal collection container No. 4 with cap, P-sagittate, with round label type 2 affixed (ASIAKIZAI Co., Tokyo, Japan).

2.3. DNA extraction and 16S rRNA sequencing

Fecal samples were stored at -80 °C until processing, and genomic DNA was extracted using the NucleoSpin Microbial DNA (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. All extracted DNA samples were quantified by fluorescence using Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific, Massachusetts, USA) and purified using the Agencourt AMPure XP (Beckman Coulter, CA, USA). Sequencing libraries were prepared using the 16S (V3-V4) Metagenomic Library Construction Kit for NGS (Takara Bio, Kusatsu, Japan). The first PCR amplification was performed using the primer pair 341 F (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGA-CAGCCTACGGGNGGCWGCAG-3′) and 806 R (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAGGGACTACHVGGGTWTCTAAT-3′) with Illumina adaptor overhang sequences. The second PCR amplification was performed using the Nextera XT Index Kit v2 (Illumina, San Diego, USA). Sequencing libraries were purified using the Agencourt AMPure XP (Beckman Coulter) and quantified by fluorescence using the Quant-iT dsDNA Assay Kit (Thermo Fisher Scientific). Clonal clusters of the libraries were generated and sequenced on a MiSeq system (Illumina) with the MiSeq Reagent v3 kit in 2×250 bp mode.

Raw sequences were filtered using QIIME2 (version: 2021.11) [14] and were demultiplexed by per-sample barcodes and Illumina-sequenced amplicon read errors were corrected by DADA2 and clustered into operational taxonomic units (OTU) at 99 % identity using the VSEARCH. Taxonomy was classified using the GreenGenes 99 % OTUs database (version 13_8). A rarefaction curve was generated after random sampling up to 10,000 sequences in each sample. Alpha diversity metrics were calculated for each sample at the sampling depth. To test the association for each taxonomy and species, low-abundance species were first filtered and then analyses were conducted using 258 OTUs (shared in more than 50 % of samples).

2.4. Statistical analyses

By 75 g OGTT, we initially compared characteristics between subjects with normal glucose tolerance (bNGT) ($n = 114$) at baseline and those with impaired glucose tolerance (bIGT) ($n = 18$) at baseline **(**Fig. 1**, Analysis1)**. Specifically, according to the WHO criteria [12], NGT was defined as a fasting plasma glucose level < 110 mg/dL and a 120-minute plasma glucose level < 140 mg/dL. In the present study, IGT encompassed individuals not classified as having neither a diabetic pattern nor NGT, incorporating the WHO's definitions of IGT and Impaired Fasting Glucose (IFG) [12] **(**Fig. 1**, Middle)**.

Next, we compared the characteristics of subjects who consistently remained with NGT at both baseline and one year later (NRN, $n = 108$) with those who consistently remained with IGT at both baseline and one year later (IRI, $n = 12$) after excluding subjects transitioning from NGT at baseline to IGT one year later (NTI, $n = 6$) and those transitioning from IGT at baseline to NGT one year later (ITN, $n = 6$) **(**Fig. 1**, Analysis1-2)**.

Finally, after excluding subjects with bIGT ($n = 18$), we included only subjects with bNGT in analyses and compared NRN ($n = 108$) and NTI ($n = 6$) **(**Fig. 1**, Analysis2)**.

Data were shown as mean ± standard error of the mean (SEM) for normal distribution variables, and as median and 25th–75th percentiles for skewed distribution variables. Comparisons between bNGT and bIGT as well as between NRN and NTI for levels of serum triglycerides, HbA1c, and plasma glucose were performed using the Mann-Whitney *U* test with an unpaired *t*-test used for the other parameters. Both alpha and beta diversities were calculated using QIIME2 (version: 2021.11) [14]. Unweighted UniFrac distance metrics were obtained to generate principal coordinate analyses (PCoA). The community structure of bNGT *vs.* bIGT, NRN *vs.* IRI, and NRN *vs.* NTI were compared by measuring Shannon and Observed Features. The *mixOmics, plsda.res,* and *ggplot2* from the R-package (version 4.3.2) were used to calculate and plot the Partial Least Squares Discriminant Analyses (PLS-DA). Statistical differences between bNGT and bIGT as well as between NRN and NTI at different taxonomic assignments were calculated using Multi-factor analyses (MFA) with criteria of $p < 0.05$ and FDR < 0.1. Statistical differences at the genus levels were calculated using MFA between bNGT and bIGT as well as between NRN and NTI with criteria of $p < 0.05$ and FDR < 0.1. MFA was performed on MicrobiomeAnalyst [15].

Spearman's rank correlation analyses were used to examine the nature of associations between taxa of gut microbiota and clinical indices in subjects with bNGT using the R-package (version 4.3.2), employing the *cor* function, *Circlize* and *ComplexHeatmap* from R-package to generate the heatmap. Other statistical analyses were performed using JMP version 15.0.0 (SAS Institute Inc., Cary, NC, USA). Statistical significance between the two-tailed level set at $p < 0.05$.

3. Results

3.1. Analyses 1: bNGT vs. bIGT

3.1.1. Characteristics of subjects

Using a 75 g OGTT, we initially divided the characteristics between subjects with bNGT (n = 114) and those with bIGT (n = 18). Significant differences in anthropometric, clinical, and biochemical parameters were observed between bNGT and bIGT at baseline (Supplementary Table S1). Age in bIGT was higher than bNGT (bNGT 55.3 ± 1.2, bIGT 63.2 ± 3.0, $p = 0.02$), while no significant differences were observed in height (bNGT 159.8 ± 0.90, bIGT 157.7 ± 2.26, $p = 0.40$), body weight (bNGT 62.9 ± 1.15, bIGT 64.0 ± 2.89, $p = 0.73$), BMI (bNGT 24.5 ± 0.33, bIGT 25.0 ± 0.84, $p = 0.64$), or waist circumference (bNGT 88.2 ± 0.88, bIGT 90.9 ± 2.21, $p = 0.25$), respectively. Systolic blood pressure (bNGT 131.7 ± 1.75, bIGT 132.4 ± 4.40, $p = 0.88$) and diastolic blood pressure (bNGT 77.2 ± 1.06, bIGT 76.5 ± 2.66, $p = 0.80$) also did not significantly differ between two groups. Biochemical parameters, the levels of total cholesterol (bNGT 204.4 ± 3.5, bIGT 209.0 ± 8.8, $p = 0.63$), HDL-cholesterol (bNGT 61.0 ± 1.5, bIGT 56.8 ± 3.9, $p = 0.31$),

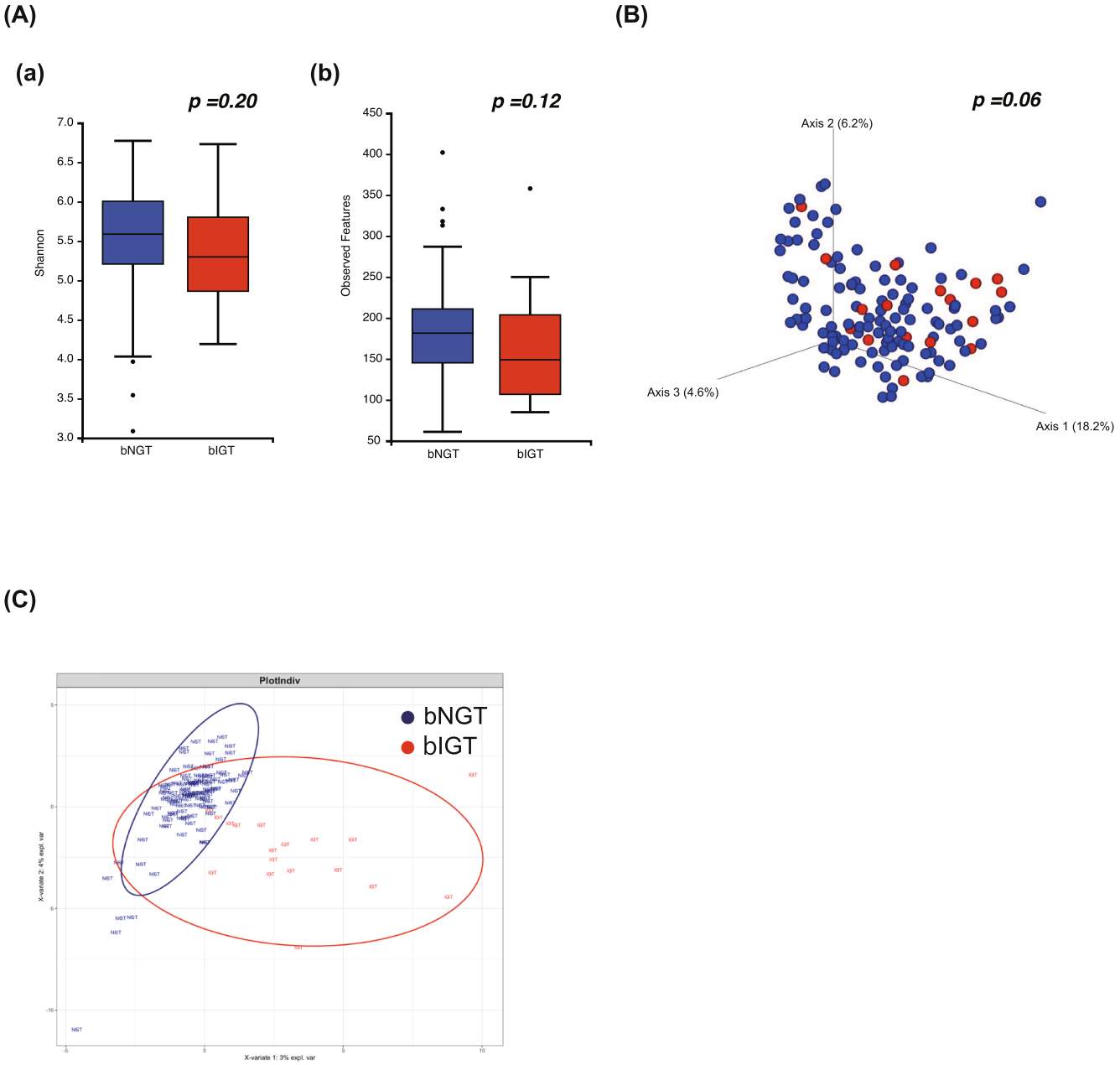

Fig. 2. Diversity and composition of gut microbiota in subjects studied of bNGT and bIGT at baseline. **(A)** Alpha diversity assessed by **(a)** Shannon and **(b)** Observed Features indices were compared between bNGT and bIGT. The Kruskal-Wallis test assessed the statistical significance between two groups. Graphics were made using QIIME2 (version 2021.11) [28]. *$p < 0.05$. **(B)** Principal component analyses plot of microbiota based on unweighted UniFrac distances shows beta diversity between bNGT and bIGT. Permutational multivariate analyses of variance (PERMANOVA) were employed to compare differences between two groups. **(C)** Partial Least-Squares Discriminant Analyses (PLS-DA) of the comparison of gut microbiota. The PLS-DA score plot illustrates the differences in gut microbiota profiles between bNGT and bIGT. Each point represents the composition of gut microbiota in each subject, and the 95 % confidence ellipses demonstrate the group differentiation based on the PLS-DA model. The explained variance is based on X-variate (normalized bacterial abundances). bNGT; normal glucose tolerance at baseline (blue) (n = 114); bIGT; impaired glucose tolerance at baseline (red) (n = 18). Graphics were made using the R-package (version 4.3.2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and LDL-cholesterol (bNGT 121.1 ± 3.1, bIGT 122.5 ± 7.7, $p = 0.87$) also did not differ significantly.

However, serum triglycerides level was notably different, with the bIGT group showing an increased median level compared to bNGT (bNGT 93.5 (67.0–143.0), bIGT 144.5 (93.0–193.5), $p = 0.03$). Serum HbA1c level was also significantly different, with higher levels observed in the bIGT group (bNGT 5.5 (5.3–5.7), bIGT 5.8 (5.6–6.0), $p = 0.0003$).

In the 75 g OGTT, bIGT showed significantly higher levels of fasting plasma glucose as well as postprandial glucose exclusion at all time points (fasting: bNGT 81.0 (76.0–85.3), bIGT 90.0 (82.0–105.0), $p < 0.0001$, after 30 min: bNGT 136.0 (121.0–150.3), bIGT 166.5 (148.0–191.3), $p < 0.0001$, after 60 min: bNGT 126.5 (97.8–150.3), bIGT 198.5 (154.8–217.0), $p < 0.0001$, after 90 min: bNGT 109.0 (92.8–130.0), bIGT 193.5 (150.8–212.5), $p < 0.0001$, after 120 min: bNGT 104.5 (85.0–120.0), bIGT 152.0 (146.0–171.5), $p < 0.0001$, respectively).

3.1.2. Analyses of gut microbiota

Comparative analyses of gut microbiota between bNGT and bIGT showed no significant differences in both alpha and beta diversity measurements, but detailed multivariate analyses revealed some differences in bacterial composition. Alpha diversity showed no significant differences between bNGT and bIGT as assessed by the Shannon ($p = 0.20$) **(**Fig. 2**A-a)** and Observed Features ($p = 0.12$) **(**Fig. 2**A-b)** with the Kruskal–Wallis test. Beta diversity showed no difference ($p = 0.06$) in the comparison between bNGT and bIGT as demonstrated by Principal Coordinates Analyses (PCoA). The percentage of variation explained by Axis 1 was 18.2 %, by Axis 2 was 6.2 %, and by Axis 3 was 4.6 %, with the visual observation being confirmed by the PERMANOVA test **(**Fig. 2**B)**.

Importantly, the PLS-DA performed to visually compare gut microbiota profiles revealed distinct clustering patterns between bNGT and bIGT, while there was a bit of overlap in the 95 % confidence ellipse regions **(**Fig. 2**C)**. Furthermore, detailed analyses using MFA at the genus level revealed that *Clostridium* was significantly abundant in bIGT compared to bNGT. (Log2FC = −0.02, $p = 0.0007$, FDR = 0.067).

3.2. Analyses 1–2: Consistent NGT (NRN) vs. Consistent IGT (IRI) at baseline and after one year

3.2.1. Analyses of gut microbiota

Alpha diversity showed a significant difference between NRN (n = 108) and IRI (n = 12) as assessed by the Observed Features with the Kruskal-Wallis test (Shannon: $p = 0.05$, Observed Features: $p < 0.05$) **(Supplementary Fig. S1A-a, b)**. Beta diversity showed a significant difference ($p = 0.04$) in the comparison between NRN and IRI as demonstrated by PCoA. The percentage of variation explained by Axis 1 was 18.5 %, by Axis 2 was 6.2 %, and by Axis 3 was 4.6 % with the visual observation being confirmed by the PERMANOVA test **(Supplementary Fig. S1B)**.

3.3. Analyses 2: NRN vs. NTI

3.3.1. Subject characteristics

Comparing anthropometric, clinical, and biochemical parameters between NRN (n = 108) and NTI (n = 6) revealed no significant differences in most of the clinical parameters **(**Supplementary Table S2**)**. No significant differences were found in age (NRN 54.9 ± 1.3, NTI 62.3 ± 5.4, $p = 0.18$), height (NRN 159.7 ± 0.95, NTI 162.6 ± 4.04, $p = 0.49$), body weight (NRN 62.7 ± 1.16, NTI 67.0 ± 4.94, $p = 0.40$), BMI (NRN 24.4 ± 0.35, NTI 26.4 ± 1.49, $p = 0.20$), or waist circumference (NRN 87.9 ± 0.91, NTI 93.6 ± 3.85, $p = 0.15$). Systolic blood pressure (NRN 131.5 ± 1.80, NTI 135.2 ± 7.83, $p = 0.65$) and diastolic blood pressure (NRN 77.2 ± 1.12, NTI 77.2 ± 4.77, $p = 0.99$) were also comparable between two groups. Biochemical parameters revealed no significant differences in total cholesterol (NRN 204.4 ± 3.5, NTI 205.0 ± 14.9, $p = 0.97$), HDL-cholesterol (NRN 61.0 ± 1.6, NTI 60.7 ± 6.7, $p = 0.96$), LDL-cholesterol (NRN 120.9 ± 3.1, NTI 125.3 ± 13.2, $p = 0.74$), or triglycerides (NRN 94.0 (67.0–145.0), NTI 85.5 (66.0–137.5), $p = 0.68$), respectively.

However, NTI was significantly higher in the value HbA1c (NRN 5.5 (5.3–5.6), NTI 5.9 (5.6–6.2), $p = 0.008$) compared to NRN. In the 75 g OGTT, significant differences were observed at the point after 30 min (NRN 134.5 (120.3–150.0), NTI 151.5 (137.5–195.8), $p < 0.05$). However, no significant differences were noted at other time points (fasting: NRN 80.5 (75.3–85.0), NTI: 86.0 (79.8–93.5), $p = 0.08$, after 60 min: NRN 124.0 (97.0–145.8), NTI 145.0 (122.8–219.5), $p = 0.09$, after 90 min: NRN 108.0 (92.0–128.0), NTI 138.5 (105.3–162.8), $p = 0.06$, 120 min: NRN 104.5 (85.0–120.0), NTI 111.0 (93.0–123.8), $p = 0.47$, respectively).

3.3.2. Analyses of gut microbiota

Comparative analyses of gut microbiota between NRN and NTI showed no significant differences in both alpha and beta diversity, but detailed multivariate analyses revealed evident differences in bacterial composition. Alpha diversity showed no difference between NRN and NTI as assessed by the Shannon ($p = 0.18$) **(**Fig. 3**A-a)** and Observed Features ($p = 0.57$) **(**Fig. 3**A-b)** with the Kruskal–Wallis test. Beta diversity showed no difference ($p = 0.77$) in the comparison between NRN and NTI as demonstrated by PCoA. The percentage of variation explained by Axis 1 was 17.7 %, by Axis 2 was 6.3 %, and by Axis 3 was 4.6 %, with the visual observation being confirmed by the PERMANOVA test **(**Fig. 3**B)**.

Notably, however, PLS-DA performed to visually compare gut microbiota profiles revealed distinct clustering patterns between NRN and NTI with the 95 % confidence ellipse regions, showing no overlap **(**Fig. 3**C)**.

Furthermore, after adjusting for age, sex, the value of HbA1c, and BMI, MFA at the genus level identified four gut microbiota genera as significantly prevalent in NTI compared to NRN: *CF231* (Log2FC = -0.003, $p = 0.000006$, FDR = 0.004), *Corynebacterium* (log2FC = -0.0002, $p = 0.00001$, FDR = 0.004), *Succinivibrio* (Log2FC = -0.02, $p = 0.0007$, FDR = 0.07), and *Geobacillus* (Log2FC = -0.00006, $p = 0.001$, FDR = 0.08) **(**Table 1**)**. Additionally, analyses conducted without covariates (crude) and with age and sex revealed that all these genera were significantly abundant in NTI compared to NRN **(**Supplementary **Table S3)**.

In subjects with bNGT (n = 114), the correlations between parameters associated with impaired glucose tolerance (circulating IRG, fasting blood glucose levels, and the value of HbA1c) and four gut microbiota genera (*CF231, Corynebacterium, Succinivibrio,* and *Geobacillus*) were analyzed using a heatmap based on Spearman's rank correlation coefficients **(**Fig. 3**D)**. The results showed a positive correlation between *Succinivibrio* and the value of HbA1c ($p < 0.01$).

Results of IRI (n = 12) and ITN (n = 6) subjects were also examined. However, there were no significant differences in both alpha and beta diversity between two groups in the present study.

4. Discussion

The major findings in the present study include distinct differences in the composition of baseline gut microbiota between NRN and NTI. Detailed analyses of gut microbiota showed no significant increase in specific gut microbiota in NRN. However, *CF231, Corynebacterium, Succinivibrio,* and *Geobacillus* were substantially abundant in NTI compared to NRN even after adjusting for age, sex, HbA1c level, and BMI.

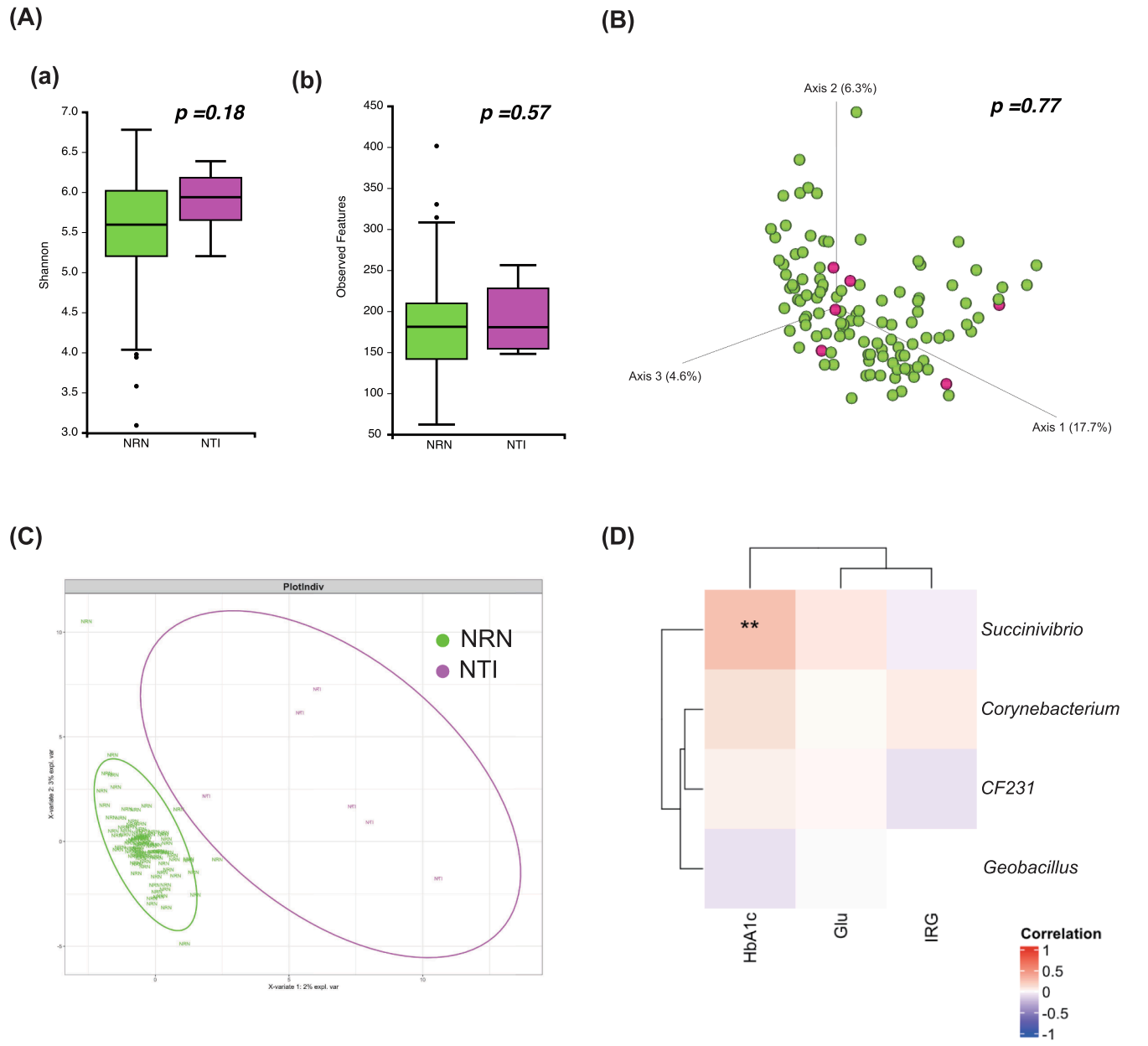

Fig. 3. Diversity and composition of gut microbiota in subjects studied of NRN and NTI at baseline and heatmap of subjects with normal glucose tolerance at baseline. **(A)** Alpha diversity assessed by **(a)** Shannon and **(b)** Observed Features indices were compared between NRN and NTI. The Kruskal-Wallis test assessed the statistical significance between two groups. Graphics were made using QIIME2 (version 2021.11) [28]. *$p < 0.05$. **(B)** Principal component analyses plot of microbiota based on unweighted UniFrac distances shows beta diversity between NRN and NTI. Permutational multivariate analyses of variance (PERMANOVA) were employed to compare differences between two groups. **(C)** Partial Least-Squares Discriminant Analyses (PLS-DA) of the composition of gut microbiota. The PLS-DA score plot illustrates the differences in gut microbiota profiles between NRN and NTI. Each point represents the composition of gut microbiota in each subject, and the 95 % confidence ellipses demonstrate the group differentiation based on the PLS-DA model. The explained variance is based on X-variate (normalized bacterial abundances). NRN; consistent normal glucose tolerance at baseline and one year later (green) (n = 108); NTI; normal glucose tolerance at baseline and impaired glucose tolerance one year later (pink) (n = 6). Graphics were made using the R-package (version 4.3.2). **(D)** Spearman's correlation heatmap showing significant correlations of gut microbiota at the genus level and selected parameters in subjects with normal glucose tolerance at baseline (n = 114). Significant correlations are shown as red (positive) or blue (negative). Graphics were made using the R-package (version 4.3.2). **$p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.1. Differences in the gut microbiota between individuals with baseline NGT (bNGT) and baseline IGT (bIGT)

A previous study showed an apparent trend of reduced diversity and imbalance of gut microbiota in IGT compared to NGT [16]. Regarding alpha and beta diversity in the present study, no significant differences were observed between bNGT and bIGT. On the other hand, both alpha and beta diversities were significantly lower in IRI than in NRN, consistent with a previous report [16].

PLS-DA visualization of differences in gut microbiota composition between bNGT and bIGT revealed distinct clustering patterns between two groups, while there was some overlap in the 95 % confidence ellipse regions. Additionally, the MFA at the genus level identified the presence of *Clostridium* in bIGT. Given that some of the *Clostridium* species decrease in patients with T2DM [14] while others conversely proliferate in IGT or T2DM as opportunistic pathogens [15], observed increase of

Table 1
Multi-factor analyses of gut microbiota composition (NRN *vs.* NTI).

Microbiota (genus)	Log2FC	St.Error	*p*-value	FDR
CF231	−0.003	0.0006	0.000006	0.004
Corynebacterium	−0.0002	0.00003	0.00001	0.004
Succinivibrio	−0.02	0.004	0.0007	0.07
Geobacillus	−0.00006	0.00002	0.001	0.08

This table displays differences in the abundance of gut microbiota identified between NRN and NTI when age, sex, the value of HbA1c, and BMI were all included as covariates in a linear model. $p < 0.05$. FDR < 0.1.
Log2FC; Log2 Fold Change represents the logarithmic fold change in the abundance of gut microbiota between two groups. A positive value indicates a higher abundance in NRN, while a negative value indicates a higher abundance in NTI.
St. Error; The standard error of the Log2FC. The *p*-value is obtained from *t*-tests.
FDR; False Discovery Rate.
NRN; consistent normal glucose tolerance at baseline and one year later (n = 108), NTI (NGT to IGT); normal glucose tolerance at baseline and impaired glucose tolerance one year later (n = 6). Data were analyzed using Multi-factor analyses of MicrobiomeAnalyst 2.0 [15].

Clostridium in bIGT could potentially be explained by the activation of opportunistic pathogens within this genus, which may subsequently contribute to glucose intolerance.

Conversely, no genera were found to be significantly prevalent in bNGT compared to bIGT, although it has been well documented that genera such as *Alistipes*[7], *Akkermansia*[8], *Bacteroides*[5] and *Faecalibacterium*[6] are significantly prevalent in subjects with NGT compared to IGT. Such a discrepancy may be attributable to geographical differences. In this sense, further studies are warranted to test the reproducibility in different geographic areas.

4.2. Profile of gut microbiota as a predictive marker for transitioning from NGT to IGT

No significant differences in alpha and beta diversity were observed between NRN and NTI. As both groups were normoglycemic at baseline, this result was within our expectation. Surprisingly, however, PLS-DA visualization of the composition of gut microbiota formed distinct clusters without overlapping in the 95 % confidence ellipsoidal regions between NRN and NTI, indicating clear compositional differences, which were never identified from diversity analyses.

After adjusting for age, sex, the value of HbA1c, and BMI, MFA at the genus level identified a higher prevalence of *CF231, Corynebacterium, Succinivibrio,* and *Geobacillus* in NTI compared to NRN. These genera may serve as predictive markers for transitioning from NGT to IGT.

CF231, related to the progression from cirrhosis to hepatocellular carcinoma [17], belongs to the family Paraprevotellaceae, which is associated with BMI [18], dyslipidemia, and glucose intolerance [19], and also shows an increase in subjects with MUO in our previous study [11].

While some species of *Corynebacterium* exert anti-inflammatory effects in humans [20], an increase in *Corynebacterium* was conversely shown to associate with T2DM [21].

Succinivibrio, associated with the risk of T2DM and obesity disease in humans [22], was found to increase in MUO correlating positively with the value of HOMA-R, levels of serum triglycerides, BMI, body weight, waist circumference, and HbA1c in our previous study [11].

Geobacillus is known to increase in patients with cholangiocarcinoma [23] and bladder cancer [24], but its clinical implication in glucose homeostasis still remains unclear.

Despite no differences except for the value of serum HbA1c and 75 g OGTT plasma glucose after 30 min in the baseline profiles between NRN and NTI, and even after adjusting for covariates to align background factors, abundance of *CF231, Corynebacterium, Succinivibrio,* and *Geobacillus* were significantly higher in NTI compared to NRN. Coupled with aforementioned previous studies that link these genera to metabolic deterioration, this finding may suggest a potential role for a line of intestinal bacteria in the risk of glucose intolerance.

We do acknowledge several critical limitations in the present study. First, due to its cross-sectional nature for some analyses, it is not possible to infer causality between specific profiles of gut microbiota and abnormalities in glucose homeostasis. Second, the relatively small sample size limits the power to detect changes in the gut microbiota, necessitating further validation in larger sample populations. Considering the impact of the relatively small sample size, we also evaluated statistical significance using an FDR < 0.1 in addition to a $p < 0.05$ to reduce the likelihood of incidental findings. This approach is beneficial to considerably enhance the reliability of our results. Furthermore, it should be noted that Kumejima residents, a remote island in the southernmost part of Japan, exhibit similar trends among residents in overall lifestyle habits, including daily food consumption and physical activity. This might be advantageous for obtaining reliable data on the profile of gut microbiota.

5. Conclusion

This study identified specific gut microbiota that may predict the transition from NGT to IGT in residents of a remote island with similar lifestyle trends. The findings suggest that differences in gut microbiota profiles could be used to predict the transition from NGT to IGT. Although the applicability of our findings needs to be validated by extensive studies, results may provide unique insight into early screening and intervention in subjects with a higher risk of T2DM.

6. Authors' relationships and activities

The authors declare no competing interests.

7. Funding.

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8. Author contributions.

All authors contributed to the study's conception and design. Biological sample and analyses as well as subject data collection were undertaken by T.U. and M.U., and K.H. Statistical analyses were performed by T.U. under the supervision of K.N. The first draft of the manuscript was written by T.U. and M.T. T.U., M.T., K.Y., S.O., M.U., K. H., Y.N., A. T., MI.M., A.A., S.M., M.I., MA.M., K.N., and H.M reviewed and edited the final manuscript. Project administration was undertaken by T.U., K. Y., M.U., K.H., MI.M., A.A., MA.M., and H.M. supervised the study. All authors read and approved the final manuscript.

9. Informed Consent Statement

Written informed consent was obtained from all of the subjects involved in the present study.

CRediT authorship contribution statement

Tsugumi Uema: Writing – review & editing, Writing – original draft, Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Mari Tsukita:** Writing – review & editing, Writing – original draft, Visualization. **Shiki Okamoto:** Writing – review & editing, Methodology. **Moriyuki Uehara:** Writing – review & editing, Investigation. **Ken-ichiro Honma:** Writing – review & editing, Investigation. **Yoshiro Nakayama:** Writing – review & editing. **Atsuko Tamaki:** Writing – review & editing. **Minoru Miyazato:** Writing – review & editing, Project administration, Investigation. **Asuka Ashikari:** Writing – review & editing, Investigation. **Shiro Maeda:** Writing –

review & editing, Methodology, Investigation. **Minako Imamura:** Writing – review & editing, Investigation. **Masayuki Matsushita:** Writing – review & editing, Supervision, Project administration, Funding acquisition. **Koshi Nakamura:** Writing – review & editing, Supervision, Methodology, Formal analysis. **Hiroaki Masuzaki:** Writing – review & editing, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. The sponsors were not involved in the study design; collection, analyses, or interpretation of the data; the writing of this manuscript; or the decision to submit the manuscript for publication. The authors, their immediate families, and any research foundations with which they are affiliated have not received any financial payment or other benefits from any commercial entity related to the subject of this article.

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Appendix A. Supplementary data

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