Raspberry consumption: identification of distinct immune-metabolic response profiles by whole blood transcriptome profiling

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Trial Registration
This trial was approved by the Laval University Ethic Committee on January 3rd, 2018 and is registered to the Clinical Trials registry (NCT03620617).

Keywords
Raspberry; metabolic syndrome; multi-omics; gut microbiota; immunity; gene expression; phospholipids; clustering approaches

Abstract
**Background.** Numerous studies reported that diets rich in phenolic compounds are beneficial to human health, especially for immune-metabolic conditions, yet these effects and underlying mechanisms are not well defined.

**Objectives.** The main goal of this study was to investigate the architecture of the inter-individual variability of the immune-metabolic response to raspberry consumption, by identifying distinct sub-groups of participants sharing similar transcriptomic signatures.

**Methods.** The 24 participants assigned to the treated arm of a randomized controlled trial, and at risk of developing metabolic syndrome, received 280g/day of frozen raspberries for 8 weeks. RNAseq data from whole blood assessed at weeks 0 and 8 were used to identify sub-groups of responses to raspberry consumption, by using partial least-squares discriminant analysis (PLS-DA) and hierarchical clustering. Changes in clinical features, metabolic parameters, plasma metabolites and gut metagenomics were compared between the resulting sub-groups.

**Results.** Transcriptomic-based clustering regrouped the initial 24 study participants into two significantly different sub-groups of response to raspberry consumption, with 13 participants being defined as responders and 11 as non-responders. Following raspberry consumption, a significant decrease in plasma triglycerides, total-cholesterol and C-reactive protein was found in the responder sub-group, as compared to the non-responder sub-group. Two major components composed respectively of 100 and 220 genes were further identified by sparse PLS-DA as those better discriminating responders and non-responders. Functional pathways related to cytokine production, leukocyte activation and immune response were significantly enriched with discriminant genes. Factor analysis revealed that the first metabolomic factor mostly composed of decreasing triglycerides and increasing phosphatidylcholines was significantly higher in responders, as compared to non-responders. Analysis of gut metagenomic data revealed differences between responders and non-responders prior to the intervention and distinct modulations, notably
regarding Firmicutes and Actinobacteria phyla. **Conclusions.** The discrimination analysis carried out in the present study based on transcriptional changes following raspberry consumption was able to identify two divergent sub-groups of participants, which were further identified as responders and non-responders, according to their immune-metabolic and gut metagenomic responses. In the context of precision nutrition, this holistic approach represents a promising framework to tackle the issue of inter-individual variability in the understanding of the impact of foods or nutrients on immune-metabolic health.
**Introduction**

Incipient metabolic dysregulations can manifest themselves in a broad range of intermediate phenotypes, all converging with time toward metabolic syndrome (MetS), a multifactorial pathophysiological condition clustering several interrelated immune-metabolic abnormalities that increase the risk of type 2 diabetes and cardiovascular disease (1). Environmental diversity and genetic heterogeneity contribute to both the variability of immune-metabolic intermediate phenotypes and the complexity of MetS etiology (2). Metabolic syndrome is then the result of a complex interplay between genetic and environmental factors reflecting an unsuitability of the dietary environment regarding the organism ability to maintain immune-metabolic homeostasis. The genetically conditioned nutritional requirements of humans have been shaped by millions of years of a hunting and gathering lifestyle, which involved significantly higher intakes of plant food components, primarily fiber and phytochemicals, than at present (3). Thus, despite remaining controversies regarding the exact role of several factors, such as microbiota or low-grade inflammation, in the pathophysiology of the MetS and the underlying causal mechanisms, the health benefits of increased consumption of plant foods are not disputed (4). Clinical trials carried out with metabolically disturbed subjects, highlighted beneficial effects of raspberry (Rb) consumption on immune-metabolic health (5-10). In a randomized controlled trial, subjects at risk of developing metabolic syndrome received 280g/day of frozen Rb for 8 weeks (11). Potentially interrelated modulations of differentially expressed genes and functional pathways of lipid and phospholipid metabolism were observed in the Rb group. However, the great heterogeneity observed in the clinical results obscured central trends and, therefore, complicates the elucidation of potential mechanisms of action of Rb compounds. Herein, we hypothesized that transcriptional changes occurring throughout the intervention may form a basis for a discrimination of distinct sub-groups of immune-metabolic responsiveness to Rb consumption. To address this hypothesis, a transcriptomic-based clustering approach was conducted to
identify sub-groups with distinct gene expression features, and then clinical phenotypes, as well as global metabolome and gut metagenomic profiles of these emerging clusters were compared.
Materials and methods

Study design and data collection

Data used in the present study were obtained from participants of a two-arm parallel group, randomized, controlled trial on Rb consumption (11). The objective of this clinical trial was to investigate the effects of an 8-week Rb supplementation on metabolic syndrome features, blood transcriptome and plasma metabolome in subjects presenting slight metabolic disorders. The trial was registered at clinicaltrials.gov as NCT03620617 and approved by the Université Laval Ethics Committee (CER-Université Laval 2017–218). Written informed consent was obtained from participants. Details on the methodology used have been previously described (11). A total of 59 participants with overweight or abdominal obesity, and with slight hyperinsulinemia or hypertriglyceridemia, were randomized to consume either 280g/day of frozen Rb (n=24) or maintain their usual diet (n=25). Briefly, data collection took place between 2018 and 2019 and included people aged 18–60 years, from the greater Quebec City metropolitan area. Data were collected through questionnaires and physical examination that included the collection of biological samples at weeks 0 and 8, before and after the Rb supplementation, respectively (11). Metabolic parameters including plasma insulin, glucose, lipids and lipoproteins, blood pressure were assessed following standard clinical protocols and are detailed elsewhere (11). Only data from the 24 participants enrolled in the Rb-treated arm were used in the present analysis.

Statistical analyses

The normality of distribution of all metabolic and clinical variables was assessed using skewness and kurtosis, and none of the variables needed transformation before analysis. Descriptive characteristics between groups were presented as mean ± standard deviation. Chi-square tests for categorical variables and analyses of variance (general linear model, type III sum of squares) for continuous variables were used to seek for inter-group differences in baseline characteristics and in changes between follow-up and
baseline levels. The mixed procedure in SAS with special provisions for repeated measures that incorporated data from the two visits was used to examine the effects of group, visits and their interaction on clinical variables. Data were adjusted for age, sex, body mass index (BMI) and baseline values. A p-value lower than 0.05 was considered for statistical significance in all analyses, which were performed using SAS version 3.8 (SAS Institute, Cary, NC, USA).

**RNA sequencing**

RNA sequencing was carried out in blood samples of the 24 participants of the experimental group to assess the transcriptomic changes following the intervention (11). Briefly, fasting blood samples were collected into PAXgene tubes (Qiagen, Valencia, CA, USA) at weeks 0 and 8, total RNA was extracted with the RNeasy Mini Kit (Qiagen) following manufacturer’s instructions and the quality of RNA was evaluated with the 2100 Bioanalyzer (Agilent, Santa Clara, CA, US). RNA sequencing was performed at the McGill University and Genome Quebec Innovation Centre. Briefly, library preparation was carried out using the Illumina NEB stranded mRNA library preparation kit (Illumina, San Diego, CA, US) and sequencing was performed on the Illumina NovaSeq6000 S4 platform (Illumina) using 100bp paired-end reads. Raw reads were trimmed for length (n=50), quality (phred33 score ≥ 30) and adaptor sequence using Trim Galore (v0.6.5), a wrapper tool around Cutadapt (v1.15) and FastQC (v0.11.9). Trimmed reads were pseudo-aligned to the GRCh38 human reference transcriptome using kallisto (v0.46.2) and transcript abundance was estimated with default parameters and 100 bootstraps, and reported in estimated counts (12). Data normalization and automatic filtering of estimated counts were performed with edgeR v3.28.1 (13). Differential transcript expression across the Rb group between weeks 8 and 0 was determined using a generalization of a paired t-test implemented in the quasi-likelihood framework of edgeR. Most variable transcripts, defined as those transcripts showing differential expression at non-corrected p-value lower
than 0.05 were retained. Pre- and post-supplementation counts per million (cpm) of most variable transcripts were used for clustering analysis.

**Transcriptomic-based clustering analysis**

The identification of sub-groups of participants with distinct transcriptomic response signatures to the Rb supplementation was carried out by combining a classification method, the partial least squares-discriminant analysis (PLS-DA), and hierarchical clustering analysis (HCA). The PLS-DA is a supervised method that uses multivariate PLS regression to discriminate the membership of samples to a given class using multidimensional quantitative explanatory variables. Herein, we used PLS-DA to discriminate transcriptomic profiles of participants between pre- and post-supplementation visits. The performance of the PLS-DA model was evaluated by estimating the balanced error rate (BER) and the optimal number of components was determined. In order to reduce the number of uninformative genes, the classifier was built on the most variable genes found in our previous study (1384 genes) (11). Unsupervised HCA of latent variables form the final PLS-DA model was then used to seek for sub-groups of participants (clusters) with distinct transcriptomic response profiles. HCA was performed using Euclidean distance and the Ward agglomerative method in the pvclust R package, which computes approximately unbiased p-values (AU) for each of the clusters via multiscale bootstrap resampling (n=1000 replications). Distinct clusters were considered to be strongly supported by data when AU ≥ 95%. We finally performed a sparse variant of the PLS-DA (sPLS-DA) on the newly identified clusters of participants to optimize discrimination and to determine the subsets of genes that best discriminate the different sub-groups of response. The sPLS-DA performs sample classification and variable selection in a one-step procedure, and the optimal number of genes was set during the tuning process with 10-fold cross validation and 10 repeats. The multilevel extension of both PLS-DA and sPLS-DA was used to exploit the paired structure of the transcriptomic data obtained before and after the Rb supplementation, at weeks 0 and 8,
respectively. Both classification algorithms were implemented using the mixOmics R package (v6.12.1).

The clinical relevance of the newly created clusters was further evaluated by comparing the responsiveness to the Rb supplementation between response sub-groups. The functional significance of the most discriminative genes was explored by pathway enrichment analysis using the clusterProfiler v3.16.0 R package (14) and the Gene Ontology Biological Processes (GO-BP) pathway database.

**Targeted serum metabolome profiling**

Plasma samples collected at weeks 0 and 8 were sent to the Analytical Facility for Bioactive Molecules at the Hospital for Sick Children in Toronto, Canada. The quantitative analysis of 630 metabolites from 26 biochemical classes was performed in paired blood samples from the 24 participants before and after the Rb supplementation with the MxP® Quant 500 kit for targeted metabolic profiling (Biocrates Life Sciences AG, Innsbruck, Austria), as previously described (11). These metabolites included mostly PC, TG, acylcarnitines, lysophosphatidylcholines, sphingomyelins, ceramides, dihydroceramides, hexosylceramides, dihexosylceramides, trihexosylceramides, cholesteryl esters, diglycerides, amino acids, amino acid related, bile acids, biogenic amines and carboxylic acids. The analysis of metabolites combined flow injection analysis (FIA) with liquid chromatography-based triple quadrupole mass spectrometry (LC-MS/MS), and was performed in an Agilent 1200 series HPLC chromatograph coupled to a SCIEX QTrap 5500 mass spectrometer. Metabolite data were preprocessed using the MetaboAnalystR package (v3.0) (15). First, metabolites with constant or single values across samples were removed, and non-informative signals were filtered out based on the interquartile range. A total of 383 metabolites that passed quality filters were normalized by quantile normalization, log-transformed and scaled by Pareto scaling. Delta values between weeks 0 and 8 were obtained for each metabolite and included as input data in principal component (PCA) and principal factor (PFA) analysis that were used to reduce the large number of correlated metabolites into clusters of fewer uncorrelated factors. First, PCA was performed to
determine the minimal number of components explaining most of the variance. These components explaining more than 10% of variance were retained for further analyses. Second, PFA was then used to assign metabolites with a factor pattern ± 0.5 to a given factor. Finally, metabolite factor scores were calculated for each individual as the sum of metabolites multiplied by their respective factor patterns. PCA and PFA were performed using princomp and factor procedures, respectively, in SAS 3.8 (SAS Institute, Cary, NC, USA).

**Fecal metagenomics**

Stool samples were collected at weeks 0 and 8 during the intervention using the EasySampler® Stool Collection Kit (Alpco). Samples were stored at -20°C until the delivery at the research center within 24 hours, where they were stored at -80°C until analysis. Global variation of the fecal microbiota from the 24 participants was analyzed at the beginning (week 0) and at the end (week 8) of the intervention. Whole metagenome shotgun sequencing analysis was used to determine the Rb impact on gut microbial composition. Bacterial DNA extraction was performed using a combination of physical cell-disruption (bead beater) and silica column purification (Qiagen QIamp DNA Stool Mini Kit). DNA concentrations were evaluated spectrophotometrically using a NanoDrop ND-1000 (Thermo scientific). For whole genome sequencing of each DNA sample, shotgun libraries were generated, and sequencing was performed at the Genome Quebec Innovation Centre using the HiSeq 2500 technology generating an average of 200 million 125 bp paired-end reads per lane (up to 8 lanes by run on HiSeq 2500). Paired reads from fastq files were trimmed and quality filtered (with a cutoff ≥Q20) using Trimmomatic (16) and further mapped to the human hg38 genome using Bowtie2 (17) to remove the host-origin reads. Host-decontaminated reads were taxonomically annotated at the species level using SLIMM (18). To generate abundance estimation, SLIMM uses coverage information of reference genomes to gain accuracy in taxonomic profiling. We removed bacterial species with total abundance lower than 0.1% and not seen
more than 3 times in the entire dataset. In order to adjust for differences in library sizes across samples, data were rarefied before calculating alpha diversity analysis. The alpha diversity of bacterial species was investigated by calculating the Shannon index, which is strongly influenced by richness and rare species and the Simpson’s reciprocal index, which gives more weight to evenness and common species. Additionally, dissimilarity of microbial communities between Rb sub-groups was calculated on clr (centered log-ratio) transformed data using PLS-DA at weeks 0 and 8. Difference in alpha diversity between groups was compared using Wilcoxon signed-rank test. Detection of differentially abundant taxa between Rb sub-groups was performed with a DESeq2 model adjusted for subject (19). P-values were adjusted for multiple testing using Benjamini-Hochberg false discovery rate correction in DESeq2. A p-value lower than 0.05 was considered as being statistically significant. All statistical analyses on metagenomic data were performed under R (www.r-project.org).
Results

Hierarchical clustering of raspberry-supplementation response sub-groups

Results from the transcriptomic-based multilevel PLS-DA classifier showed that the first two components of the model explained 39% and 35% of the total variance in expression levels, respectively (*Figure 1a*). As expected, the observed discrimination between weeks 0 and 8 when using the ungrouped cohort of participants was, however, not enough to make a definite distinction between pre- and post-supplementation visits. HCA was then applied on the two main latent variables derived from the PLS-DA model, revealing a total of four distinct clusters at AU > 95% (*Figure 1b*). Two of these clusters regrouped 13 matched participants with well-differentiated gene expression profiles at weeks 0 and 8, while the other two clusters regrouped 11 participants with mixed transcriptomic profiles. As shown in *Figure 1b*, new sub-groups of participants were accordingly re-coded as responders i.e. the 13 participants whose gene expression profiles at weeks 0 and 8 were significantly discriminated, and non-responders i.e. the 11 participants whose gene expression profiles were mixed. Newly created sub-groups were then projected into the PLS-DA subspace spanned by the first two components for descriptive purposes (*Figure 1a*). Further, sPLS-DA was used to enhance sub-group discrimination by determining the optimal number of genes contributing the most to such discrimination. After sPLS-DA model tuning, two components composed of respectively 100 and 220 genes were identified (*Table S1*). As shown in *Figure 1c*, the two first components of the optimized model explained 38% and 34% of variance in gene expression levels, with an improved discrimination between pre- and post-supplementation in the sub-group of responders. This relevant group discrimination is also illustrated in *Figure 1d*, with pre- and post-supplementation visits being completely discriminated within the group of responders, but mostly mixed in the sub-group of non-responders.

Baseline and longitudinal changes of immune-metabolic phenotypes between Rb sub-groups
The discrimination of participants into distinct sub-groups did not create major dissimilarities regarding baseline characteristics, and no significant differences between responders and non-responders were observed for age, sex, anthropometric, physiologic or metabolic variables (Table 1). According to BMI, among responders and non-responders respectively, 4 and 8 subjects had obesity (BMI > 30 kg/m²), 8 and 2 were overweight (BMI between 25 and 30 kg/m²), and one in each group was in the normal BMI range (BMI < 25 kg/m²). The mean BMI was 29.3 kg/m² (ranging from 24.7 – 37.7 kg/m²) for responders and 32.8 kg/m² (ranging from 22.6 – 43.7 kg/m²) for non-responders. However, although not statistically significant, BMI and sex ratio differences between groups were not negligible, as well as the ensuing differences in terms of waist circumference (Table 1). Changes in anthropometric and metabolic parameters from baseline to the end of the 8-week intervention are depicted in Table 2. Significant group (responders vs. non-responders with regards to their transcriptomic signatures) and group-by-visit interaction effects were noted for plasma triglycerides (TG) (p_{group}=0.03 and p_{interaction}=0.04) and total cholesterol (total-C; p_{group}=0.03 and p_{interaction}=0.01) with TG and total-C decreasing in the responder group following the Rb supplementation while increasing in the non-responder group. We also noted significant group, visit and group-by-visit interaction effects for plasma C-reactive protein (CRP) levels (p_{group}=0.04, p_{visit}=0.04 and p_{interaction}=0.03) with non-responders exhibiting significantly increased CRP levels whereas a slight decline was noted among responders. Although not significant, a trend for a decrease in LBP in the responder group versus an increased in non-responders was observed (p_{interaction}=0.08). Interestingly, significant correlations were found between the change in plasma CRP levels and specific metabolites, mainly lipid species, such as TG and PC, among others (Table S1). A sole significant negative correlation was also found between changes in plasma CRP concentrations and changes in the expression of HDAC7, a gene coding for the enzyme histone deacetylase 7 (r=-0.45, p=0.03). No significant differences between sub-groups were found with regards to changes in daily dietary intakes (data not shown).
Identification of most discriminant genes and functional pathway analysis

Among the 100 and 220 genes identified as being the most discriminative between responder and non-responder sub-groups from sPLS-DA (Figure 1d), a ranking was obtained according to their loading weights, representing the contribution of each gene to sub-group discrimination in a given component. Top-ten most discriminative genes from components 1 and 2 derived from the sPLS-DA model are shown in Figure 2a and Figure 2b, respectively. The magnitude of gene expression changes in component 1 was constantly higher among responders, as compared to non-responders (Figure 2c). Similarly, the down-regulation of genes in component 2 was persistently more important in the sub-group of responders (Figure 2d). A broader representation of the top-100 most discriminative genes on each component illustrates the direction and the magnitude of gene expression changes, with most of genes in the component 1 being up-regulated following the Rb supplementation (Figure 2e), and those in the component 2 exhibiting a marked down-regulation (Figure 2f). Further functional pathway analysis with genes from components 1 and 2 revealed a total of 18 pathways significantly enriched, most of them were related to cytokine production, leukocyte activation and immune response. Top-ten significantly enriched pathways are shown in Figure 2g and the total number of significantly enriched pathways can be accessed in Table S3.

Principal component analysis of metabolites

As most of the plasma metabolites measured showed collinearity, PCA and PFA were used to reduce their large number into independent factors. Three factors explaining most of the total variance were kept, so that the contribution of each group of metabolites to these factors illustrated the main trends they followed during the Rb supplementation. Thus, three main factors were extracted which, together, represented about 66.4% of the observed variance in the sample set. Most of the variance (40.6%) was revealed by factor 1, composed of 63 metabolites, and whose factor loading membership is outlined in Figure 3a. Metabolites
positively contributing to factor 1 were mainly phosphatidylcholines (PC) (50.8%), whereas negative contributors were mainly TG (44.4%). Overall, PC levels increased in the responder group following the Rb supplementation, and TG levels decreased, whereas the opposite trend was found in the non-responder group (Figure 3b). On the other hand, factors 2 and 3 explained 13.4% and 12.3% of the total variance, and were composed of 56 and 40 metabolites, respectively. Metabolites contributing to factors 2 and 3 were more scattered and negative contributions were negligible. On the whole, mostly diacylglycerols, bile acids, lysophospholipids, TG, PC, cholesterol esters, glycosylceramides and sphingolipids contributed to the second factor, whilst diacylglycerols, PC and TG predominantly composed the third factor. The complete list of metabolites contributing for each factor is depicted in Table S3. Factor 1 was found to be significantly associated with sub-group discrimination (p=0.001), being significantly higher in responders than in non-responders, while the second and third factors were less effective in discriminating response sub-groups (Figure 3c). In view of the lipid-related profile of factor 1 and its significant association with sub-group discrimination, we investigated its relationship with the significant decrease observed in plasma TG and total-C in responders following the Rb supplementation. As expected, a significant an inverse correlation was found between factor 1 and plasma TG levels in the total group including responders and non-responders (r=-0.68, p=2x10^{-4}). By contrast, factor 1 did not correlate with plasma total-C levels (r=-0.24, p=0.26) (Figure 3d).

**Analysis of fecal microbiota**

A PLS-DA of bacterial taxonomy derived from metagenomic data on the sub-groups previously identified based on transcriptomic data revealed a clear separation between responders and non-responders (Figure 4a). Microbiota alpha diversity was calculated on sub-groups throughout the intervention. Baseline Shannon's diversity index was higher in non-responders, although not significantly, when compared to responders (Figure 4b). However, it exhibited a marked but not significant decrease in non-responders
following the Rb consumption (p=0.74), while the opposite trend was observed among responders (p=0.18), whose index tended to increase in response to the supplementation. The Simpson's reciprocal index showed similar trends at baseline and in response to the intervention (Figure 4c). The relative abundance of Actinobacteria was significantly lower in responders than in non-responders at week 0 (q=0.001), and significantly decreased in non-responders from week 0 to week 8 (19.4% to 8.1%; q=0.048) (Figure 4d). The relative abundance of Firmicutes was significantly higher in responders, as compared to non-responders at week 0 (q=6x10^-6), and significantly decreased from week 0 to week 8 in the group of responders (65.6% to 50.4%; q=0.0006) (Figure 4d). Accordingly, the Bacteroidota-to-Firmicutes ratio was statistically lower in responders than in non-responders at week 0 (1.4 vs. 2.5; p=0.02), and it decreased following the intervention in non-responders (2.5 vs. 1.6; p=0.05) (Figure 4e).
Discussion

Previous clinical trials have reported positive effect of Rb on immune-metabolic health of metabolically disturbed subjects (5-10). Lastly, despite the absence of significant metabolic amelioration in the treated arm following the supplementation, a substantial inter-individual variability in metabolic responses was observed (11). Using data collected in the same cohort of individuals, we report herein the results of an analysis which allowed the discrimination of the participants into two sub-groups based on their changes in transcriptional profiles in response to raspberry consumption. That way, the subgroups of individuals identified demonstrated divergent responses, with a sharp improvement of the lipid profile in the group called responders (i.e. individuals with similar differentiated transcriptomic responses to the intervention), depicted by a marked decrease of both TG and total-C levels, as compared to non-responders (i.e. individuals with mixed transcriptomic responses to the intervention), whose pre-existing altered immune-metabolic state was rather aggravated following raspberry consumption as suggested by increases in plasma TG, total-C and plasma CRP concentrations. Overall, these findings point toward the liver as being divergently affected between sub-groups. Trough synthesis and secretion of very low-density lipoproteins (VLDL), the liver controls both TG and cholesterol levels, and it is also a major site of LDL catabolism. In addition, CRP is produced by hepatocytes, predominantly under transcriptional control by the cytokine IL-6 (20). Herewith, it suggests that the underlying mechanisms responsible for the divergence of the metabolic response to Rb consumption may fall under liver function, at least partly.

Refining our comprehension of the underlying mechanisms, transcriptomics, metabolomics and metagenomics brought new perspectives from which observing these changes. From metabolomics, results with PC and TG echoes the aforementioned lipid metabolism discrepancy, leading again, to the liver metabolism as a potential diverging node between sub-groups, since hepatic phospholipid homeostasis, and notably an adequate PC to phosphatidylethanolamine ratio, is critical in maintaining
liver integrity, supporting lipoprotein secretion and mitochondrial function (21). The liver is, indeed, the main site of choline metabolism, where it is found predominantly as PC (22). Interestingly, serum lipidomic profiles of subjects with metabolic syndrome who consumed 300g/day of berries (comprising 100g of Rb) for 8 weeks, underwent changes regarding several lipids including PC, TG, phosphatidylethanolamines and cholesterol esters (9).

Metagenomic results brought to light a distinct microbiota shift between sub-groups, characterized in responders by Firmicutes depletion following the raspberry consumption, and in non-responders by a reduction of the Bacteroidota-to-Firmicutes ratio and Actinobacteria abundance. Results from human studies regarding the abundance of Bacteroidota and Firmicutes and their relationship to metabolic disorders and obesity have been inconsistent (25-27). Nonetheless, both the reduction of Firmicutes abundance observed in responders following the intervention, and the decreased of the Bacteroidota-to-Firmicutes ratio found only in non-responders, are in agreement with previous findings underscoring an association between Firmicutes abundance and metabolic disorders (25, 28, 29). Likewise, the reduction of Actinobacteria abundance in non-responders is in line with their metabolic deterioration (30). On the whole, human studies reported that the consumption of berries, including Rb, modulates the microbiota composition, and notably the relative abundance of Firmicutes species, improving plasma lipid profile and reducing low-grade inflammation (7, 31, 32). Interestingly, a recent epigenome-wide analysis also revealed that subjects with obesity and a low Bacteroidota-to-Firmicutes ratio presented hypomethylation of the HDAC7 promoter in both whole blood and adipose tissue, compared to subjects with a higher ratio (33) In our study, we found a negative correlation between changes in HDAC7 gene expression and plasma CRP levels in response to raspberry consumption and significant increase in circulating CRP concentrations in individuals of the non-responder group. Whether changes in HDAC7 gene expression
were accompanied by variations in the methylation profile and/or if this played a role in the increased plasma CRP levels observed in non-responders merit further investigation.

Still, it should be noted that bioactive compounds from edible plants have been studied in regards of their inhibitory action on \textit{HDAC1}, another member of the histone deacetylase family. \textit{HDACs} are implicated in the regulation of macrophage inflammatory responses, as well as of dendritic cell and T cell development and function (34). Concretely, two of these bioactive compounds, nonanoic and 2-decenoic acid, inhibited \textit{HDAC1} with potency comparable to that of a neuroleptic drug (35). Nonanoic acid has been identified in Rb (36) and it could, along with other Rb constituents, have contributed, as a result of metabolic discrepancy of the said compounds between groups (microbiota and/or gene-related), to the immune-metabolic divergence in responders and non-responders that arose throughout the intervention, and more specifically to the modulation of \textit{HDAC7} expression. Indeed, since the gut microbiota has been reported to be related to whole blood \textit{HDAC7} gene methylation in subjects with obesity, it could effectively, depending on its composition, metabolize components, such as nonanoic acid, and thus modulate their potential effects on the host.

\textit{HDAC7} is a transcriptional repressor essential for B cell development (37). But more specifically, \textit{HDAC7} by facilitating T-cell receptor signaling, regulates the apoptotic response of developing thymocytes (38) and CD8+ T-cell differentiation (39). However, thymic development of T-cells seems to be highly sensitive to metabolic perturbations, since the mitochondrial content of T-cells decreases throughout their maturation, due to an autophagic clearance of superfluous mitochondria (40). Interestingly, \textit{MPC1}, a gene coding for a protein known as mitochondrial pyruvate carrier 1, was found among the most contributing genes to sub-group discrimination in the first component derived from sPLS-DA. \textit{MPC1} is part of an heterodimer responsible for transporting pyruvate into mitochondria, and has also been reported to be involved in T-cell maturation and immune regulation (41). More generally, mitochondrial pyruvate import
has been shown to be essential for plasma cell longevity (42). *HDAC7* and *MPC1* could be the emerged part of the immune modulations observed following the Rb consumption, potentially attributable to phytochemical or volatile compounds, such as nonanacoid acid, whose metabolization may have differed between sub-groups due to a pre-existing microbial discrepancy and may have reshaped intestinal microbial ecosystem in favor of some ecological niches and disfavor of others, leading to the modulations aforementioned. Nevertheless, the degree of causality between these variables remains impossible to establish with the present data.

In the second component, *SEC14L4*, one the most contributing, discriminating genes appears to be functionally linked to PC metabolism. The phosphatidylinositol transfer protein encoded by this gene is highly similar to the one encoded by the *Saccharomyces cerevisiae* *SEC14* gene, and most of the knowledge about it comes from yeast research (43). Accordingly, SEC14-superfamily proteins present an inositol-lipid binding capacity, but also for a broad range of alternate lipid ligands such as tocopherols, retinaldehyde or PC (44). The Rb content in sphingolipids was recently further characterised and four glycosyl-inositol-phospho-ceramides, which were not reported so far were identified (45). Considering the inositol-lipid binding capacity of SEC14-superfamily proteins and the structural similarities between these constituents and other known SEC14-ligands, we may suppose that Rb bioactive lipids could play a role in the modulation of plasma PC levels observed in the present study, maybe through a sensitive feedback loop mechanism involving SEC14 domain-containing proteins. Otherwise, we may assume that Rb consumption, as a result of a baseline difference in gut microbiota composition and function between sub-groups, induced a divergence in lipid and phospholipid liver metabolism and thus in plasma TG, total-C and PC levels, which in turn led to immune modulations mediated by SEC14 domain-containing proteins of immune cells.
Overall, subjects were clustered in two subgroups based on the divergence regarding blood transcriptional changes. We may then suppose that this clustering approach allowed us to identify subjects with divergent immune modulations in response to the raspberry consumption. Considering the strong immune regulatory role of the gut microbiota, we may speculate that the prebiotic potential of Rb indirectly exerted a significant influence on this discrimination. In addition, the pre-existing microbial differences and distinct modulation between groups suggest microbial metabolic differences regarding prebiotic metabolism. In the prospect of seeking to assess immune-metabolic responses to a unique component, the extent of pleiotropy falls within the bounding of the pathways it affects, while in the case of whole foods, small quantities of multiple components trapped in a food matrix act in subtle and pleiotropic ways, making the response multidirectional and slight. Thus, this multi-directionality, as well as the intermeshing and intercrossing of pathways complexifies the circumscription of clear phenotypes of response. Setting out from this premise, the way the clustering process was conducted here discovered relevant dimensions of the Rb metabolic response, demonstrating that inflammatory and lipid responses to Rb consumption may vary from individual to individual.

At this point, some methodological considerations are relevant to mention in order to ensure a proper interpretation of the present findings. Part of Rb health effects has been linked to its richness in phytochemical compounds, predominantly ellagitannins and anthocyanins. However, the metabolomic analyses performed in the present study did not target phenolic compounds, and thus did not allow us to relate the immune-metabolic effects observed to potential metabotypes. Also, metagenomics data depicts allochthonous microbes (transient microbes in the fecal stream), not autochthonous microbes (mucosa-associated microbes of the colon), nor small intestinal microbiota, though they all are functionally related (46). As a consequence, our understanding of the biological mechanisms underlying Rb consumption health effects could hardly go further the bounding of the omics tools used. In conclusion, this is, to our
knowledge, this is the first attempt to explore the high heterogeneity of immune-metabolic responses to a Rb supplementation by delineating sub-groups and characterizing their metabolic, metabolomic and metagenomic changes. This comprehensive multi-omic approach allowed us to propose an interpretation of the immune-metabolic changes, providing a path towards achieving a mechanistic understanding of the human variability to whole foods.

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Statement of Ethics
The study has been approved by the Ethics Committees of Laval University. All participants signed a written informed consent before participating to this study.

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Author Contributions
V.G., C.C., P.C., D.R., G.P., A.M. and M.C.V. designed the clinical trial. P.C. was responsible for the medical follow-up. J.T.M. was in charge of data management and performed bioinformatic analyses on RNAseq and metabolomic data; J.T.M. and M.F. performed statistical analysis. T.V. was in charge of gut microbiota data analysis. M.F. drafted the manuscript; M.C.V. and J.T.M. provided major revision of the manuscript; T.V., V.G., G.P., C.C., P.C., D.R. and A.M. critically revised the manuscript. All authors read and approved the final version of the manuscript. M.F., J.T.M., and M.C.V. have primary responsibility for final content.

Conflicts of Interest

The authors declare no conflicts of interest.

References

