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The gut microbial composition in humans in hot occupational settings and the effects of drinking buttermilk

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Abstract

The principal aims of the present study were to examine the latent heat stress relieving capacity of drinking buttermilk and to observe the human gut microbiota in hot occupational condition. A human crossover intervention study with 12 volunteers was studied, each of them performed 3 hours of physical work in a heat chamber. During the working period, the volunteers were given buttermilk, water or no liquid. Rectal and saliva samples were obtained after and during 3 hours of physical work.

The results showed no significant differences in diversity indices of three treatment groups, we detected, by means of salivary cortisol level analysis, significantly differences between the Buttermilk and Dehydrated groups, as well as Water and Dehydrated groups at the last time-point (P = 0.017). When it comes to qPCR results, *Lactobacillus* and *Enterobacteriaceae* were detected in 9 (75%) and 12 (100%) subjects, with the medians of 4.039 (Buttermilk), 4.02 (Dehydrated), 4.081 (Water) and 7.407 (Buttermilk), 6.763 (Dehydrated), 7.64 (Water) log 16S rRNA gene copies/g rectal samples respectively.

The PCA score and scatter plots indicated that the microbiota differed widely between individuals with regard to both composition and diversity. While the PLS score and loading scatter plots based on cortisol, core temperature and T-RFLP data explicated that the observations separated in groups which representing three treatments of same subject. However, the relationship between the stress indicators (core temperature and salivary cortisol level) and the gut microbial diversity was not clearly shown.

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Aim and objectives

The study was a human crossover intervention study with 12 volunteers, each of them performed 3 hours of physical work in a heat chamber. During the working period, the volunteers were given buttermilk, water or no liquid. Rectal and saliva samples were obtained after and during 3 hours of physical work, respectively. The aim of this study was to investigate the possible physiological effects of drinking buttermilk on heat stress by comparing the microbial composition, diversity and quantity of selected bacterial group in terms of different treatments- buttermilk, water and no liquid ingestion. The stress marker cortisol in saliva samples was measured to assess the stress level. The level of cortisol was also correlated to the gut bacterial flora.

Introduction

Buttermilk

Apart from other traditional methods, such as rest, ventilation and using shade structures, the most widely used method to cope with heat stress in workplaces in India is drinking buttermilk. Buttermilk is a traditional drink in India and other warm climates countries (e.g. Turkey, Afghanistan, Pakistan and Sri Lanka) and is a diluted form of curd (plain yogurt). It is consumed especially during summer as it is soothing to the stomach and alleviates minor stomach upsets. It is also considered to have gastrointestinal benefits as it contains bacteria such as *Lactobacillus delbrueckii,* which was the most predominant species identified in the product. The other minor genera found included *Streptococcus*, *Aeromonas, Methylobacter, Enterococcus, Micrococcus, Ralstonia, Moraxella*, and *Flavobacter* (Sathyanarayanan *et al*., 2013).

On the other hand, buttermilk contains a high level of protein, potassium, vitamin B2 , vitamin B12 and calcium, which help in providing body with required minerals and vitamins that may be depleted on account of excessive sweat (Rajendra *et al*., 2013). Additionally, it should be stressed that according the traditional method to make buttermilk in India, a significant amount of spices are usually added, such as green chili, ginger, coriander, curry leaves, asafoetida and mustard. In some studies it is found that some spices have pharmacological application against digestive disorders (Nadkarni, 1976, Atal, 1987), for instance, ginger and its derivatives have ability to inhibit enterotoxigenic *Escherchia coli* diarrhea (Chen *et al*., 2007). The recipe of the used buttermilk can be found in Appendix 2.

Gut microbiota and health

Microbiota or microflora, is a particular term for the huge commensal microorganisms that reside external (skin, oral etc.) or internal (gut etc.) human bodies. There is a growing awareness that the gut microbiota is essential for our health, both in physiological and physiopathological point of views. Efforts are now turning to investigate the role of microbiota in psychopathology by using animal models and human trials, and a growing body of evidence has proved that interaction between microbiome and human bodies is essential for the development of nervous system and regulation of neural functions (Bercik *et al*., 2012; Grenham *et al*., 2011).

The brain communicates with the organs, including the gastrointestinal (GI) tract through several axes: the hypothalamic-pituitary-adrenal (HPA) axis and the sympatho-adrenal axis, the HPA axis regulates gut mobility, permeability (John and Timothy, 2012) and cortisol secretion which affects immune cells activity (Montiel-Castro *et al*., 2013). Therefore the activation of HPA axis caused by stress response can lead to dysfunctions in the GI tract. Stress not only alters gut microbiota composition but also affect the intestinal barrier by impaired the mucus layer which led to increased gut permeability (Maes *et al*., 2012).

Stress is a common mood disorder which can be caused by nervous, endocrinal and immunological problem. Stressor might be divided in different categories such as chemical, biological and environmental stimuli. When encounter a stressor, the human body should respond to the challenge which is called stress response. An important component of stress response is regulated by the HPA system which modulates a range of activation, resulting in the releasing of cortisol (a glucocorticoid hormone) by adrenal cortex (Kozlov and Kozlova, 2014). Then cortisol causes a variety of physiological, cognitive, and behavioral changes that are necessary for the body to adapt the stressor successfully (Sapolsky *et al.,* 2000, Erickson *et al.,* 2003, Schulkin, 2010).

Measuring cortisol level is a common way to estimate stress response, especially in the blood serum (Kochetkov *et al.,* 2008, Pikovskaya *et al.,* 1997, Sudsuang *et al.,* 1991, Evolahti *et al.,* 2006). However, there are some limitations of measuring blood serum cortisol concentration as it is an invasive, especially in epidemiological and psychological studies (Kozlov and Kozlova, 2014). Thus cortisol level in saliva is a commentary technique that enables to give information of hormonal status sufficiently, informatively, taking account that cortisol level in saliva accounts 5% of blood serum cortisol (Guazzo *et al.,* 1996), it still reflects free cortisol level with accuracy (Umeda *et al*., 1981).

Heat stress

Heat stress is a major issue in workplaces across warm climates countries. Workers who are working outdoors in agriculture, construction, mining, soldiers, and firefighters are often exposed to severe heat stress, which deteriorate the work productivity, efficiency and can threaten survival. When the ambient temperature reaches or exceeds the human core temperature of 38 °C, there are well documented physiological effects on the human body, posing risks to some organ systems and also making it progressively harder to work productively, especially physically (Bennett and McMichael, 2010). It is also found that heat stress can lead to intestinal barrier dysfunction, induce increased GI tract permeability and likely an inflammatory response (Lambert, 2009).

Microbial diversity

Microbial diversity is an important indicator of human homeostasis. A relative higher microbial diversity is usually regarded as higher resistibility to ecological disturbances (Yachi and Loreau, 1999). Low microbial diversity, in contrast, is in general related to some gastrointestinal tract diseases such as ulcerative colitis, Crohn's disease (Wang *et al.,* 2007, Dicksved *et al.,* 2008), obesity (Turnbaugh *et al.,* 2009), non-alcoholic steatohepatitis (NASH) (Pessayre *et al.,*. 2002) and in infants with atopic eczema (Wang *et al.,* 2007). Thus, studying and measuring bacterial diversity in human gut is a suitable way to investigate the ecological pattern of the microflora communities.

In this study, two diversity indices were calculated to estimate the evenness and richness of the gut microbial communities, which is Shannon- Weaver index and Simpson index respectively. The Shannon index ($H' = -\sum p_i \ln p_i$) is an overall diversity index that accounts for both species abundance and evenness, and is more sensitive to change in abundance of rare species (Magurran, 1996, Hughes *et al.*, 2001). The Simpsons index ($\mathrm{D} = \sum P_i^{\,2}$) is a dominance measure and shows the chance that two subjects selected at random will be from the same species (Hughes *et al.*, 2001). The Simpson index is usually expressed as 1- D so the index increases when diversity increases (Magurran, 1996).

Material and Methodology

Subjects

Twelve subjects (20- 25 years; 6 women, 6 men) were recruited by advertisement. All subjects were normotensive, nonsmokers, and not taking any medicines that might alter the cardiovascular or

thermoregulatory responses in heat. All women had a normal menstrual cycle, were not taking oral contraceptives, and were tested in the early follicular phase (days 2-10). Subjects were all within normal range of BMI 18.5-25 (Anonymous, 2012). The subject was required to abstain from strenuous exercise for at least 48 hours prior to the test. A standardized breakfast (egg sandwich) was consumed in the morning and about 500ml of water to ensure normal hydration state. On arrival, the subject visited the toilet prior to the test.

Methodology

The test were performed in the climate lab at the Department of Design Studies (Lund University, Lund). The subject performed medium workload (Table 1), such as loading bricks, stepping and biking, for 3 hours in the hot controlled environment (around 35 degrees, 60% Relative Humidity) with three conditions: dehydrated, water and buttermilk (200 ml room tempered drink every 20 minutes). The three experimental trials were separated by at least one week and were randomized for the cross-over study.

Four saliva samples (approximately every 60 minutes during the test and one after the test person had woken up and brushed teeth, before consuming the egg sandwich and water) and one rectal sample of each subject were collected. Saliva samples were obtained by using the "Salivette Cortisol, code blue" (Sarstedt, Germany) in terms of quick and hygienic sampling (Hellhammer *et al.,* 1987). After the test, the plastic casing around the rectal probe was removed, inserted into a sterile test tube weighted and refrigerated. Both saliva and rectal samples were stored in -80 ℃ until analysis.

Table 1. Test procedure

Time	**Activities**
8:00–9:00	Subject arrives Saliva sample (note the time the test-person had woken up and brushed teeth). Subject eats a standardized breakfast (egg sandwich and 500ml of water). Visits the toilet, urine sample, rectal sensor inserted. Enters the climate chamber (37 degree Celsius, 60% RH).
Start of test	Start submaximal work task
Every 20min	Medium work task is changed. 4 tasks at rotating intervals: - Loading bricks (Metronome 30) - Stepping (Metronome 45) - Biking with legs (75W 60 rph) - Biking with hands (25W 60 rph) Saliva sample Drink provided depending on intervention (200ml, room temperature).
12:00-End of test	Exits the chamber Visits the toilet, plastic casing around the rectal probe is removed, inserted into a test tube weighted and refrigerated. Lunch provided

DNA extraction

To isolate and purify the DNA from rectal samples, the following procedures in combination with EZ1 Advanced XL (tissue kit and bacteria card; Qiagen) were used. Rectal samples were first thawed and weighed. Ten milliliter phosphate buffered saline (PBS, Oxoid, England) buffer was added in order to collect all traces of rectal samples. Vortex for 2 minutes. After centrifuging in the cold room (4℃) at 8000 rpm for

10 minutes, 500 µl PBS was added to the pellet. Twelve sterile and UV- treated glass beads (2 mm in diameter) were added into 1.5 ml tubes containing the sample. After incubating in room temperature for 10 minutes, the tubes with glass beads were shaken in an Eppendorf Mixer (Model 5432; Eppendorf, Hamburg, Germany) at 4℃ for 45 minutes. After the samples were centrifuged for 30 seconds at 3000 rpm, 200 µl supernatant was added to sample tube which was then extracted with the EZ1 Advanced XL. PBS without sample was treated in parallel as a negative control of DNA extraction. Finally, 22 µl sterile 10 x TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0) was added to elution tubes.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a specific and exponential synthesis of desired DNA region by using two specifically designed primers or oligonucleotides which are complementary to a part of the target DNA (Elizabeth *et al*., 2008). In this study, degenerative primers were used to amplify 16S ribosomal RNA (16S rRNA) genes which is highly conserved region between different bacteria species (Coenye and Vandamme, 2003). Inosine (I) in degenerative primers enable the possibility to pair with adenine (A), thymine (T), cytosine (C) or guanine (G) which increase the annealing efficiency (Watanabe *et al.,* 2001).

In this study, primer ENV-1 (5'-AGA GTT TGA TII TGG CTC AG-3') and ENV-2 (5'-CGG ITA CCT TGT TAC GAC TT-3'), annealing with 8-27 base pair respectively in *E. coli* (Brosius *et al.,* 1978), were used in non-fluorescently-labeled PCR in order to ensure the results of former DNA extraction and also to decide the volume of EB buffer in the following purification procedure. Cycling parameters in an Eppendorf Mastercycler (Hamburg, Germany) were as follows: initial denaturation at 94°C for 3 min followed by 32 cycles of denaturation at 94°C for 1 min, 50 °C for 45 s, extension 72°C for 2 min and final extension 72°C for 7 min.

Agarose Gel Electrophoresis

PCR products were electrophoresed on a 15% agarose gel in TB (Tris- Borate) buffer containing 168 µl of 3 fold GelRed Nucleic Acid staining solution at 100 V for 60 min and visualized under UV light. Two microliter GelPilot 100 base pair Plus Ladder (QIAGEN Group, Germany) was used as marker.

Terminal Restriction Fragment Length Polymorphism

As its name implies, terminal restriction fragment length polymorphism (T-RFLP) analysis measures the size polymorphism of terminal restriction fragments from a PCR amplified marker. It is a combination of DNA amplification, restriction endonucleases digestion and nucleic acid electrophoresis (Terence L Marsh, 1999). This tool allows the possibility of rapid analysis complex microbial communities as terminal restriction fragment (T-RFs) profiles and it has been often used to characterize human gut microflora (Hayashi H., *et al*., 2002; Wang M *et al.*, 2008; Karlsson C. L. J. *et al.*, 2011).

Fluorescently-labeled PCR

The 16S rRNA genes were amplified from samples using a fluorescently-labeled forward primer (FAM- ENV1) and the reverse primer ENV2 primers. FAM-ENV1 consisted of following sequences: 5'-AGA GTT TGA TII TGG CTC AG-3', fluorescently labeled with carboxyfluorescein (6- FAM) at the 5’ end. Each PCR reaction had a volume of 25 µl including, 2.5 µl of 10x PCR buffer (containing 15mM $MgCl_2$), 0.2 mM of deoxyribonucleotide triphosphate, 0.4 mM of each primer: FAM-ENV1 and ENV2 , 1.25 U of Top Taq DNA polymerase (Qiagen, Germany) and DNA template. The cycling parameters were same as described before.

Purification, DNA concentration measurement and Digestion

Products from fluorescently- labeled PCR reactions were pooled, purified and concentrated by MinElute PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA was

eluted in different volume of EB buffer (20µl, 25µl or 30µl) corresponding to the agarose gel electrophoresis results.

The concentrations of purified DNA were measured by using Nanodrop ND- 1000 spectrophotometer (Saveen Werner, Sweden). Then the purified DNA were subjected to restriction enzyme digestion with enzymes that have 4 base pair recognition site. In this study, *Msp*I (*Hpa*II) which recognizes C^CGG sites was used (Thermo Scientific, Germany). Purified PCR products were digested for 5h at 37 °C by 10 U of restriction endonuclease *Msp*I, in a total volume of 10 µl. Then the enzymes were inactivated at 65 °C for 15 min. The digestion step generated fluorescently-labeled terminal restriction fragments.

The digested amplicons were analyzed on an ABI 3130xl Genetic analyzer (Applied Biosystems, Foster city, CA, USA) with internal size standard GeneScan LIZ 600 (range 20 -600 bases) at DNA- lab (Malmö, Sweden). The output was analyzed by GeneMapper software version 4.1 (Applied Biosystems). T-RFs were resolved between 40- 580 base pairs considering background noise and accuracy. Peak detection thresholds were set as Blue and orange 40 FU, while for other dye colors were default thresholds which were 50 FU.

Quantitative PCR

Enterobacteriaceae and *Lactobacillus* were selected bacterial groups quantified by using quantitative PCR methodology. Quantitative, or real time, PCR (qPCR) is standard PCR with the advantage of detecting the amount of DNA formed after each cycle with either fluorescent dyes or fluorescently-tagged oligonucleotide probes (Higuchi R *et al*., 1992). qPCR results can be obtained faster and with less variability than standard PCR due to sensitive fluorescent chemistry and elimination of post-PCR detection procedures. The intensity of the fluorescence emitted during qPCR correlates to the amount of DNA product formed (Wong M.L *et al*., 2005).

Preparation of standard curve for qPCR

Ten microliter of selected bacteria groups, *Enterobacteriaceae* and *Lactobacillus,* were cultured in 10 ml Luria-Bertania (LB) broth with 20 µ 50 mg/ml ampicillin at 37 ℃ incubation for maximum 16 hours. The bacteria cultures were centrifuged at 8000 rpm for 3 minutes at 4°C. The plasmid DNA were purified according to the manufacturer instruction (Qiagen). The concentrations of the purified plasmid DNA were measured by NanoDrop and used to calculate 16S rRNA gene copies/µl purified DNA of each bacteria group according to the following formula:

$$copies/\mu l = \frac{[conc.]\times 10^{-9}}{\frac{(3000bp + \text{PCR amplified fragments})\times 649}{6.023\times 10^{23}}}$$

As for standard curves, *Enterobacteriaceae* and *Lactobacillus* were used. Ten-fold dilution series of purified plasmid DNA were made in elution buffer (EB). For all qPCR assays, each reaction contained 10 µl of 2× Rotor-Gene SYBR Green PCR Master Mix (Qiagen), 1 µL of each primer (Table 2), 2 µl of template DNA, and RNase-free water to the final volume of 20 µl. Samples, standards, and non-template controls were run in triplicate. The thermal cycling was performed in Rotor-Gene Q (Qiagen) with a program of 95°C for 5 min, followed by 40 cycles with denaturation at 95°C for 5 s, annealing, and elongation at 60°C for 10 and 20 s (Table 2). The fluorescent products were detected at the last step of each cycle. Melting curve analysis was made to ensure specific amplification. Absolute abundance of copy number was calculated based on standard curves using Rotor-Gene Q Series Software 1.7 (Qiagen). Number of bacteria was expressed as numbers of 16S rRNA gene copies /g weight of rectal samples.

Table 2. Oligonucleotide primers* used for qPCR

Target bacterial group	Sequence (5’-3’)	Amplicon size (bp)	Annealing and elongation time (s)	References
Lactobacillus	Lact-F: AGC AGT AGG GAA TCT TCC A Lact-R: CAC CGC TAC ACA TGG AG	341	20	4, 5
Enterobacteriaceae	Eco1457-F: CAT TGA CGT TAC CCG CAG AAG AAG C Eco1652-R: CTC TAC GAG ACT CAA GCT TGC	195	10	6

*Primers commercially synthesized by Eurofins MWG, Ebersberg, Germany

Cortisol analysis

The cortisol level in saliva samples were measured by one step immunometric competitively method which was ElectroChemiLuminiscence Immunoassay (ECLI) detection technique. The sample-antigen-antibody complexes were detected by an electro-chemical reaction which resulted in the emission of light (electrochemiluminescence) whose intensities were measured. The light intensity was inversely proportional to the concentration of cortisol in the sample. (Wild D., 2005)

In order to observe the effects of drinking buttermilk, water or dehydration on free cortisol level, a table of normal level of salivary cortisol concentration for approximately 700 adults over 4 time periods is included (Table 3).

Table 3. The normal level of salivary cortisol concentration (means± SD):

Time	Concentration (nmol/L)
8:00	14.32± 9.1
9:00	17.99± 7.156
10:00	14.6± 5.853
11:00	10.12± 5.332

Revised from Kirschbaum *et al.,* 1994 and Kirschbaum *et al*., 2000

Statistical Analysis and calculation

The relative abundance of each terminal restriction fragment (T-RFs) was calculated as the peak area of a certain T-RF divided by the total peak area of all T-RFs in the given T-RFLP pattern with fragment length detection limit of 40 to 580 base pair. The relative abundance which is p_i in the following formula, was used for calculation of Shannon and Simpson diversity indices (Magurran and Anne, 2013):

$$H' = -\sum p_i \ln p_i$$

$$1 - D = 1 - \sum P_i^2$$

The differences in microbial diversity among buttermilk, water and dehydrated treatments group were tested by using Kruskal–Wallis rank sum test. The qPCR data evaluated with Nemenyi-Damico-Wolfe-Dunn test (NDWD) for pairwise comparisons using package “coin” in the R program.

The copy number of the 16S rRNA gene per gram of rectal samples were calculated as follows:

$$\text{copy numbers per gram rectal sample} = q/(((\text{weight of rectal samples}/D_1) * D_2/200) \times 2\,\mu l)$$

Where q is the detected copy numbers from 2 µl of diluted template, D_1 and D_2 are the dilution factor which were 500 µl and 200 µl respectively, 200 µl was the volume of the elution of DNA extraction.

When it comes to cortisol data, 1- way ANOVA was used to determine significant effects among the four time points of three treatments. Likewise, significant differences between female and male subjects of four time points were also tested using 1- way ANOVA. Correlation and linear regression was used to test the relation between cortisol level and *Enterobacteriaceae* population.

For all univariate statistical evaluations, a *P* value of < 0.05 was considered statistically significant. Multivariate data analysis was performed using SIMCA-P + 12.0 (Umetrics, Umeå, Sweden) to reveal differences between the treatment group.

RESULTS

Diversity indices

No significant difference (*P* >.05) was observed in the Shannon or Simpson index of rectal microbiota between the three treatments groups (Table 4 and Fig. 1). As the plots shown, the diversity indices of water treatment group were more centered compared to buttermilk and dehydrated groups, which indicate the water treatment had less influence on subjects' microbiota diversity.

Table 4 Microbial diversity indices in rectal content of the three treatments groups; buttermilk, dehydrated and water

	Buttermilk		Dehydrated		Water	
	Shannon	Simpson	Shannon	Simpson	Shannon	Simpson
Median	2.859	0.899	2.922	0.913	2.933	0.916
Interquartile Range (IQR)	0.381	0.034	0.714	0.085	0.268	0.007

Data are expressed as median values and interquartile ranges, for Shannon index, *P*= .99 and for Simpson index, *P*= 1.

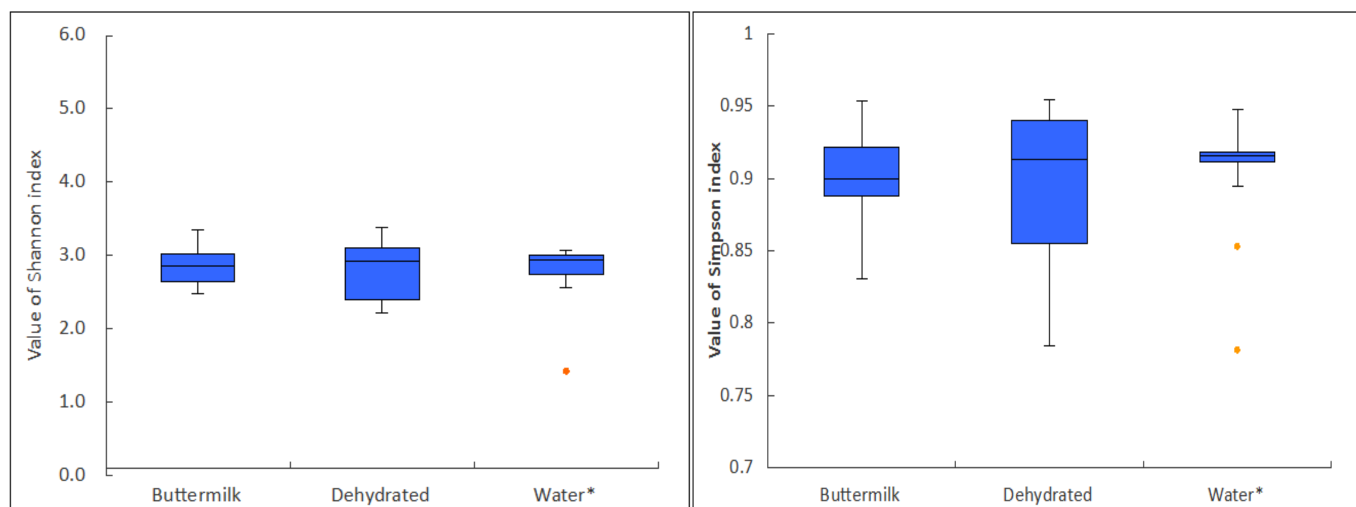


Figure 1 Shannon and Simpson indices after T-RFLP of 16S rRNA with *MspI* for digestion, generated from the rectal microbiota of subjects of three treatments group, Buttermilk (B, n=12), Dehydrated (D, n=12) and Water (W, n=12) respectively. * For Water group, orange dots were outliers.

Number of peaks

The median number of peaks after digestion with *Msp*I were 40, 41 and 43 for Buttermilk, Water and Dehydrated group respectively (Fig. 2). There was no significantly difference among the three treatment ($P > .05$).

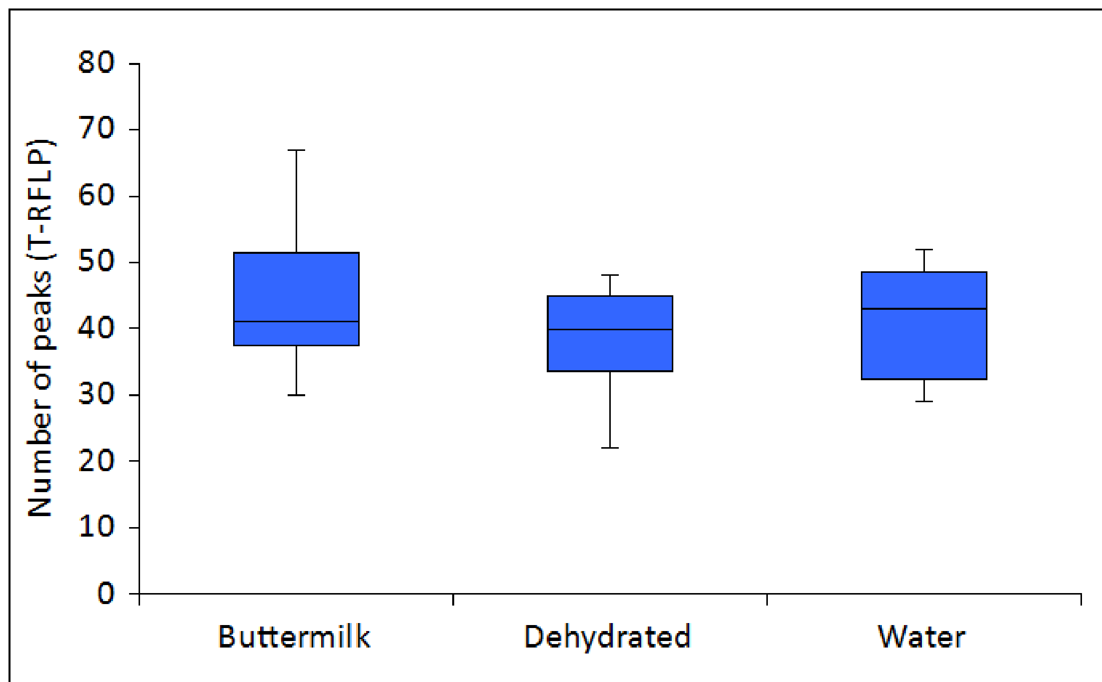


Figure 2 Medians number of peaks after T-RFLP of 16S rRNA with *Msp*I for cutting, generated from the rectal microbiota of three treatments group, buttermilk, and water and dehydrated, respectively.

Quantitative PCR

Lactobacillus and *Enterobacteriaceae* were detected with qPCR in 9 and 12 subjects respectively, with the load of 10^3 to 10^9 16S rRNA gene copies/ g rectal samples (Table 5). No significant difference ($P > .05$) between the 16S rRNA gene copies/ g rectal samples of three treatments. However, higher values of IQR were found in the *Enterobacteriaceae* population compared to *Lactobacillus* group, which suggest that the treatments had bigger influence on the population of *Lactobacillus*.

Table 5 Log 16S rRNA gene copy numbers/g rectal samples of subject harboring the analyzed bacterial taxa from different treatments detected by qPCR (Median, IQR).

	log 16S rRNA gene **copies/g rectal samples**			
	Lactobacillus		***Enterobacteriaceae***	
	Median	IQR	Median	IQR
Buttermilk	4.039	0.135	7.407	3.815
Dehydrated	4.020	0.110	6.763	3.501
Water	4.081	0.114	7.640	2.007

Saliva cortisol level

In the present study, the dehydrated group had a relative high saliva cortisol concentration at time point 4 compared with buttermilk and water treatment groups (Table 6). Additionally, the saliva cortisol level of subjects drinking buttermilk and water were all within the normal cortisol level range, while at the last time point the cortisol levels of dehydrated group were above the normal salivary cortisol level (Table 6). Significant differences between the Buttermilk and Dehydrated groups, as well as Water and Dehydrated groups at the last time-point were found ($P = 0.017$), but differences among the other three time points

were not significant. The saliva cortisol level of dehydrated treatment group had a fluctuation over 4 hours' time period while the cortisol levels of buttermilk and water treatment subjects showed a smooth decreasing trend as illustrated in Figure 7.

Table 6 Saliva cortisol levels profile from 8:00 to 11:00 of three treatments (n=12).

	Buttermilk		Dehydrated		Water	
Time	Mean	SD	Mean	SD	Mean	SD
8:00	21.38	11.43	16.04	8.74	18.04	9.70
9:00	17.20	8.71	16.33	11.57	17.28	10.45
10:00	13.43	4.05	12.55	5.86	13.33	5.55
11:00	11.79	4.17	18.44	7.72	12.05	3.98

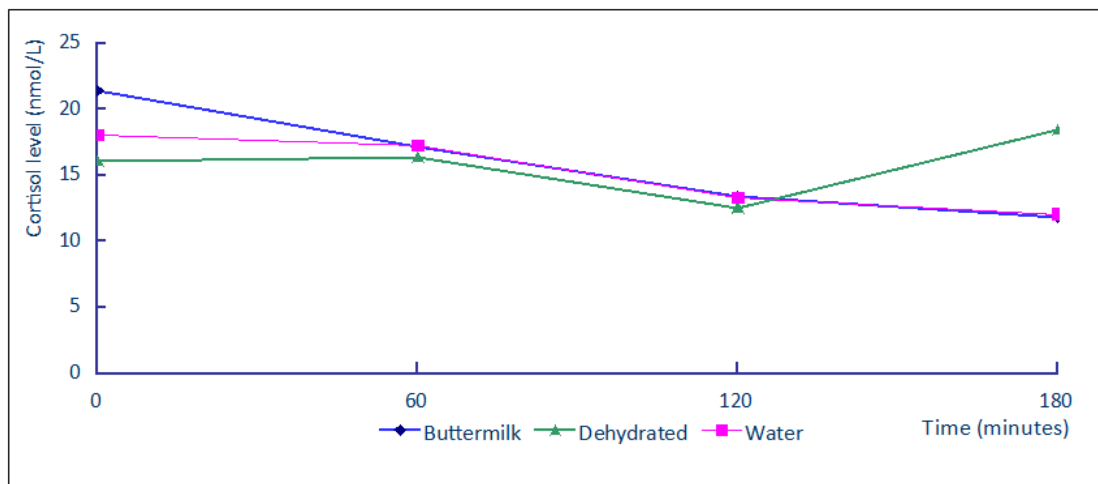


Figure 7 Saliva cortisol level profile from 8:00 to 11:00 in 12 subjects

In order to observe the potential differences of cortisol profile in gender, two salivary cortisol level profiles were created (Table 7). The data were not significantly ($P > .05$) different between male and female of same treatment. However, it can be concluded that at the last time point of the dehydrated group, the female subjects experienced a harder time in terms of high level of cortisol secreted compared with the males (Fig. 8).

Table 7 Saliva cortisol level (nmol/L) between 8:00 and 11:00 in males (n=6) and females (n=6)

Male	Buttermilk		Dehydrated		Water	
	Mean	SD	Mean	SD	Mean	SD
1	18.10	9.22	19.24	7.12	15.58	8.45
2	16.57	10.30	21.98	13.29	21.07	14.06
3	11.72	3.11	12.93	3.24	12.55	5.39
4	11.80	3.80	16.17	6.71	12.10	3.74

Female	Buttermilk		Dehydrated		Water	
	Mean	SD	Mean	SD	Mean	SD
1	24.67	13.29	13.37	9.67	20.5	11.00
2	17.83	7.73	10.68	6.43	13.5	2.88
3	15.13	4.40	12.17	8.04	14.1	6.12
4	11.78	5.05	23	8.89	12	4.71

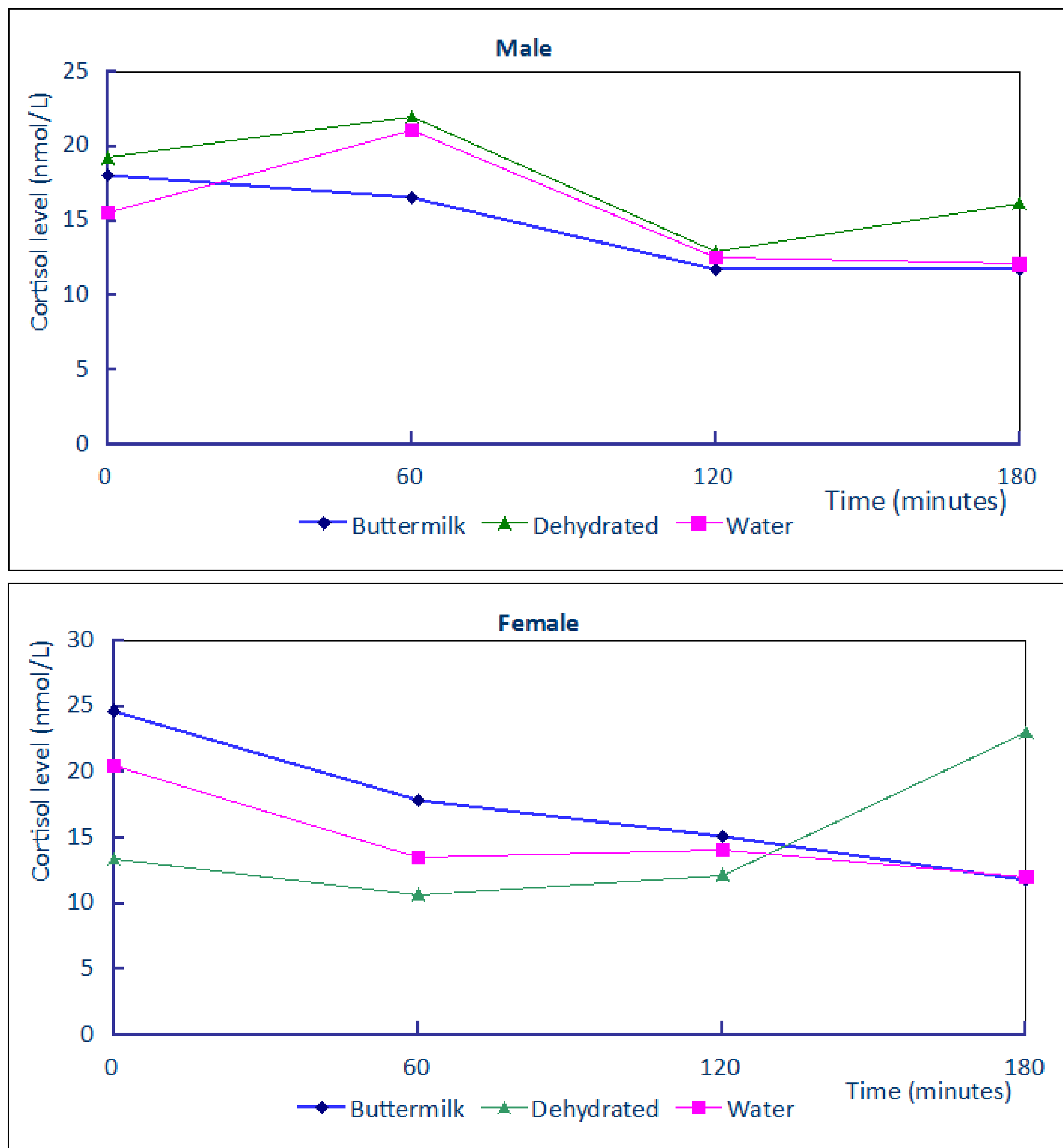


Figure 8a, 8b Saliva cortisol level (nmol/L) during 180 minutes profile in male and female subjects

Cortisol level and *Enterobacteriaceae* population

Whether the cortisol levels were correlated to the population of *Enterobacteriaceae*, correlation and linear regression test was used. For cortisol level at time point 2, time point 4 and *Enterobacteriaceae* population, the correlation were not significant (*P*= 0.334, 0.933).

Multivariate data analysis

The multivariate data analysis depict a substantial similarity or dissimilarity between different subjects and illustrate the potential relations between variables and observations. In order to have an overview, a Principle Component Analysis (PCA) score scatter plots of T-RFLP matrix was created, it clearly showed that the same subject of three treatments clustered together according the size of T-RFs (Fig. 9).

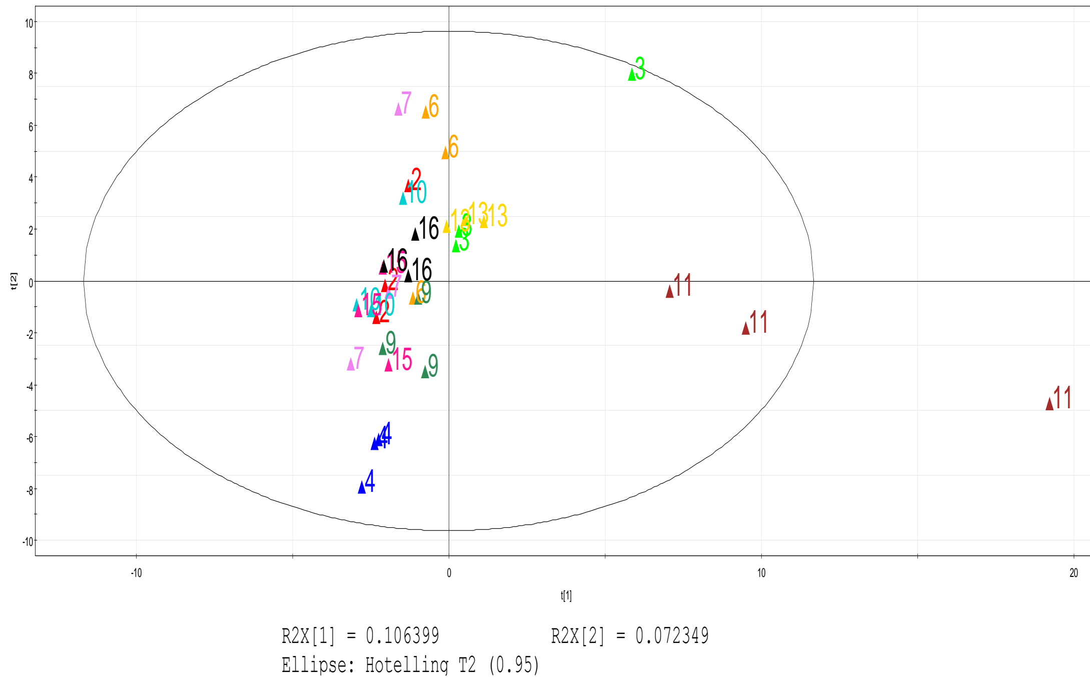
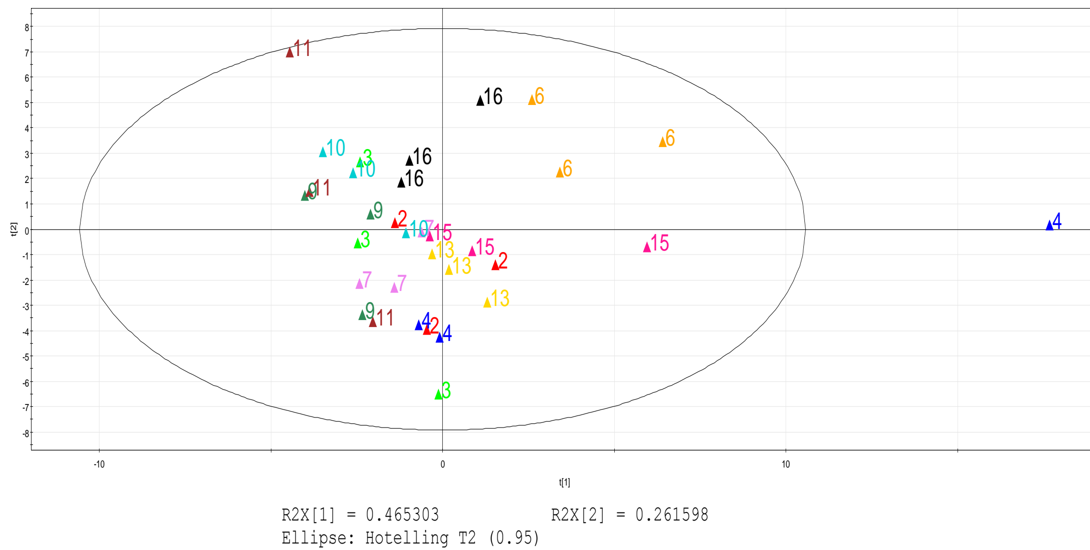


Figure 9 PCA score plot (Par scale setting) of subjects based on the T-RFLP data

PCA score scatter plot based on cortisol level, qPCR data, diversity indices as well as peak numbers of T-RFLP profile, were separated clearly in different subjects. The variables are correlated and fairly well summarized by seven variables. The scores, explaining 64.2% of the variation (Fig.10a). While the PCA loading plot explained the separation of different observations (Fig. 10b).



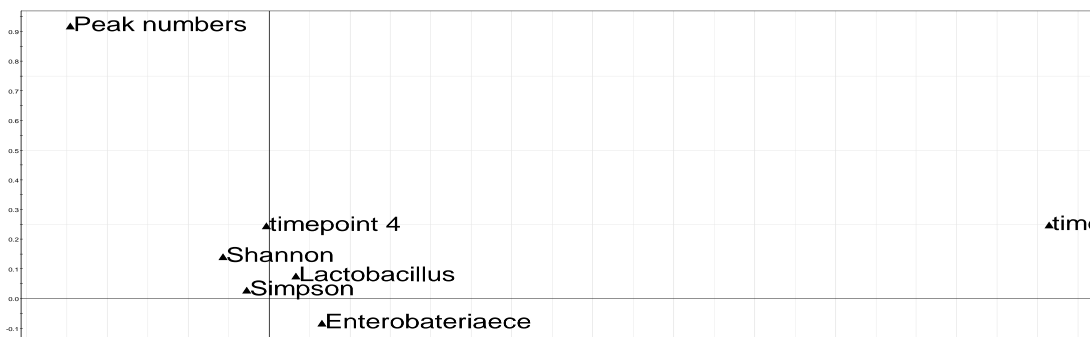


Figure 10a 10b PCA score and loading scatter plot (Scaling Par) of subjects based on Cortisol level, qPCR (*Enterobacteriaceae* and *Lactobacillus*), Diversity indices and number of T-RFLP peaks

To show the similarity and dissimilarity among observations (subjects), the observations were colored by different variables categorizes, Shannon index, Peak numbers and Cortisol level time point 4 respectively (Fig. 12, 13, 14). These three variables exerted different separation pressure towards the subjects which also explain the distribution of the loading plot (Fig. 10b). Whereas the separation of Variable Simpson, qPCR data were not very obviously (Figures not shown).

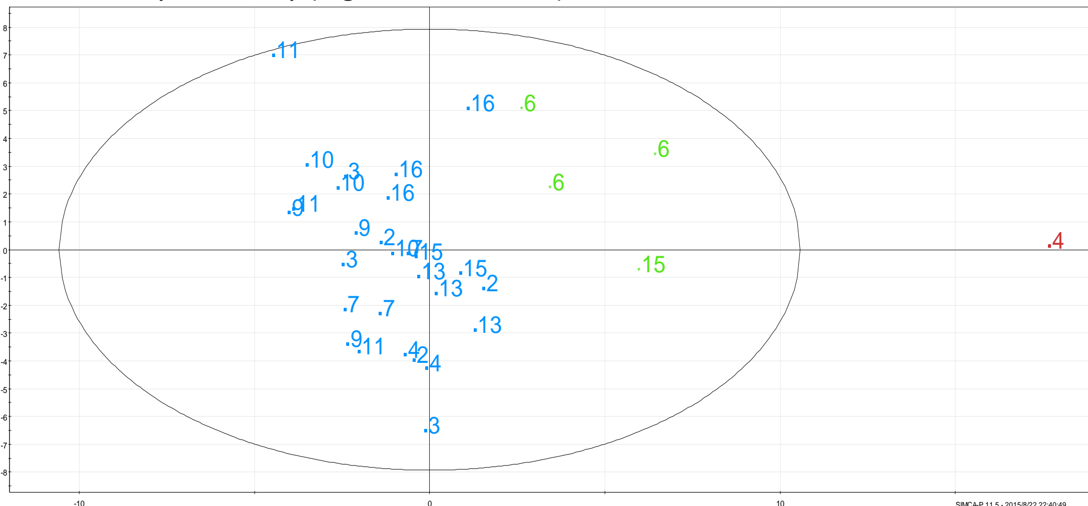


Figure 12. PCA score scatter plot colored by Peak numbers variable categories. Green data points were peak numbers between 6.2 and 33.8, blue data points were between 33.8 and 61.4, and red data point was from 61.4 to 89.

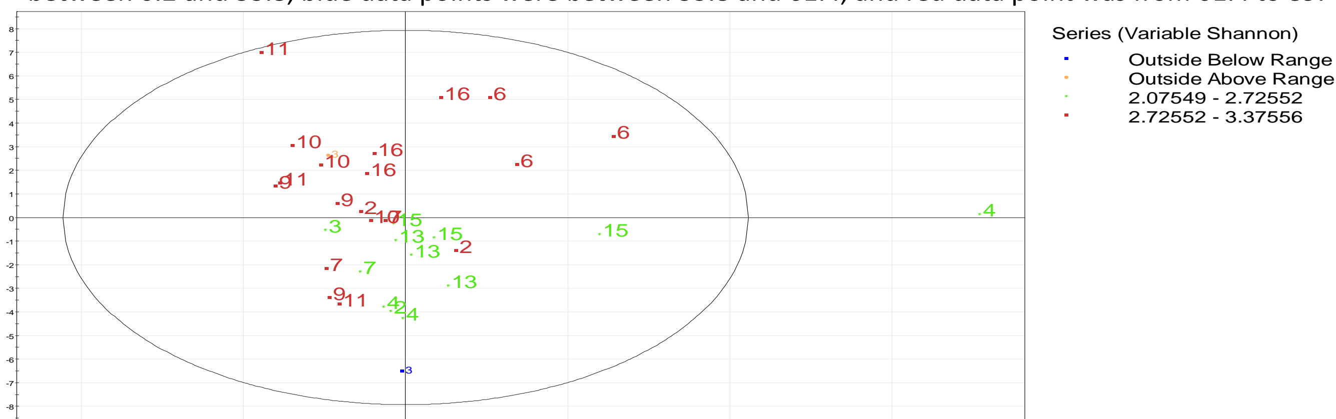


Figure 13. PCA score scatter plot colored according Variable Shannon index.

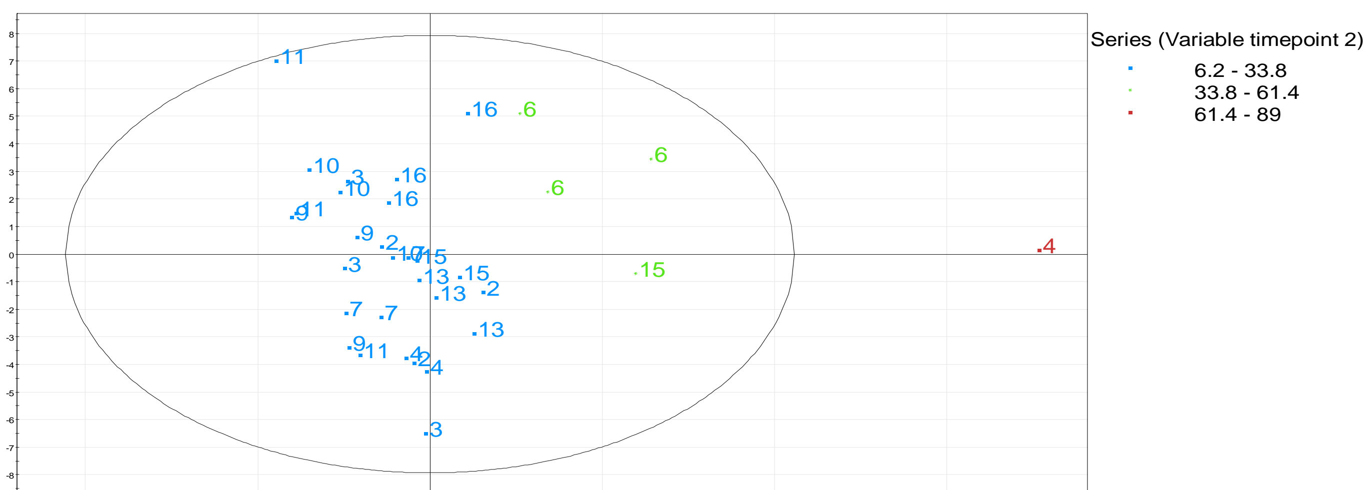


Figure 14. PCA score scatter plot colored according Variable cortisol level time point 2.

In order to investigate the relationship between the cortisol level, core temperature (which tested as indicators of heat stress) and T-RFLP data output (describing the gut microbiota diversity), two Partial Least Squares projection to latent structures (PLS) models were created.

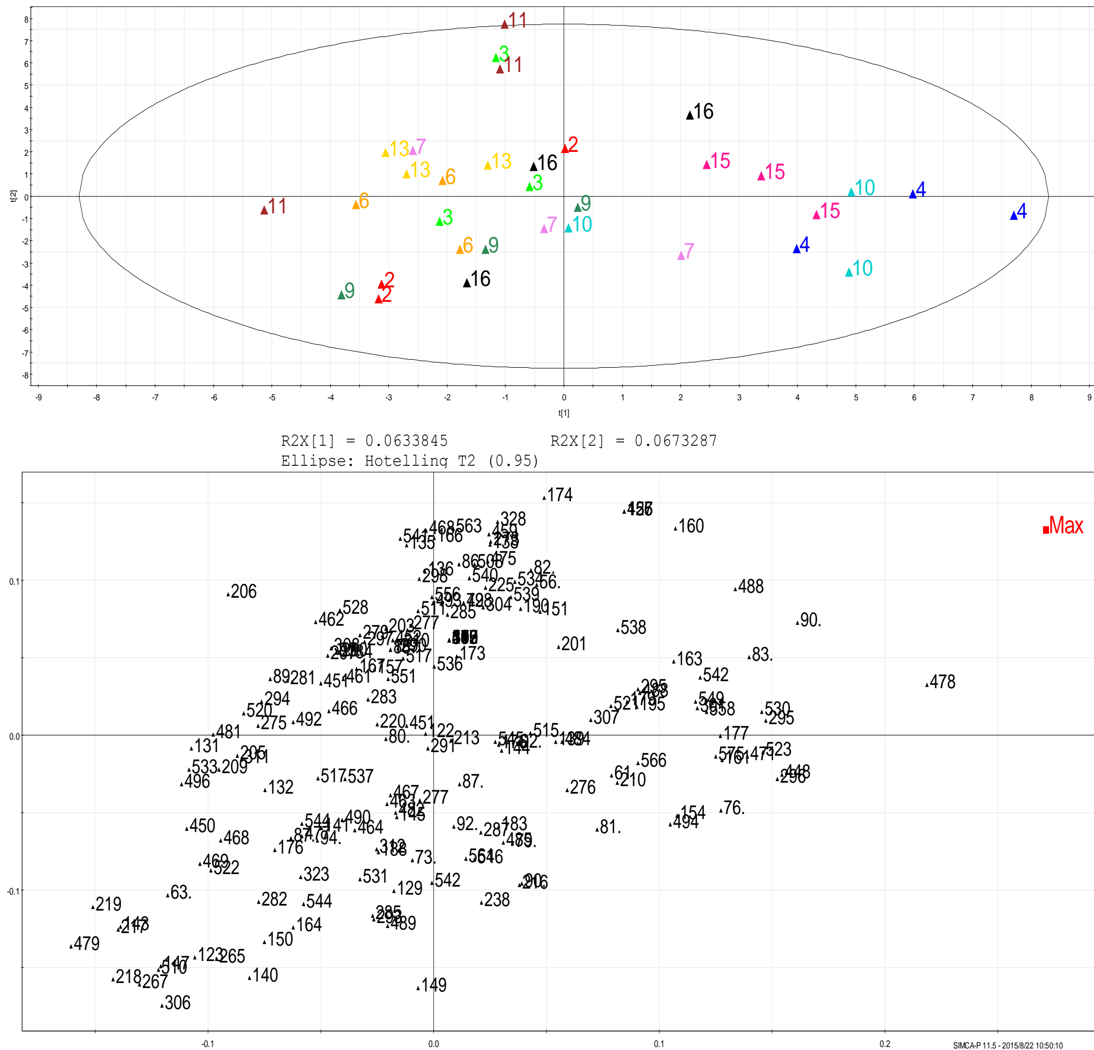
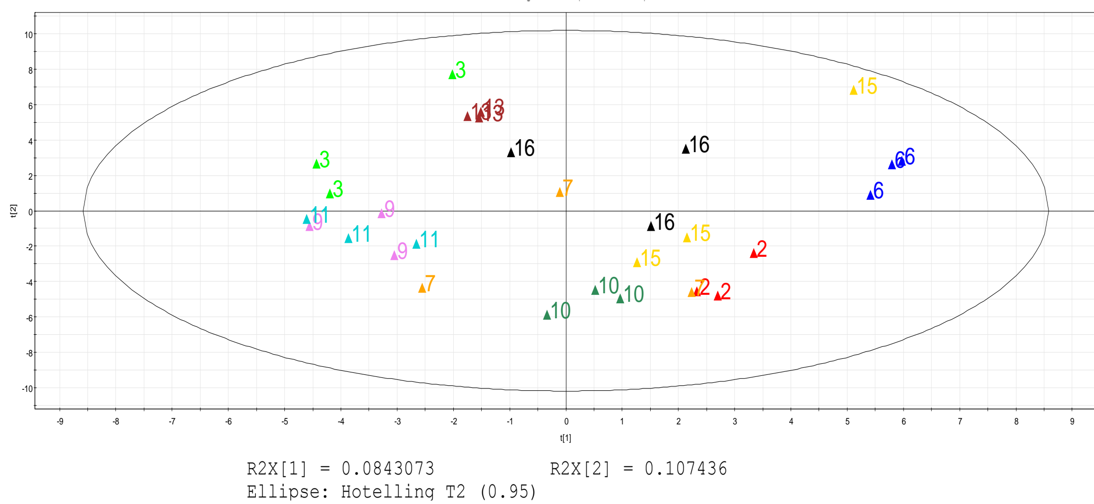


Figure 15a, b PLS score and loading plot of Max core temperature and T-RFLP data matrix

Max core temperature was positively correlated with most variables, and a shift in microbiota can be observed. However the trend of shifting was not very obviously (Fig. 15b).



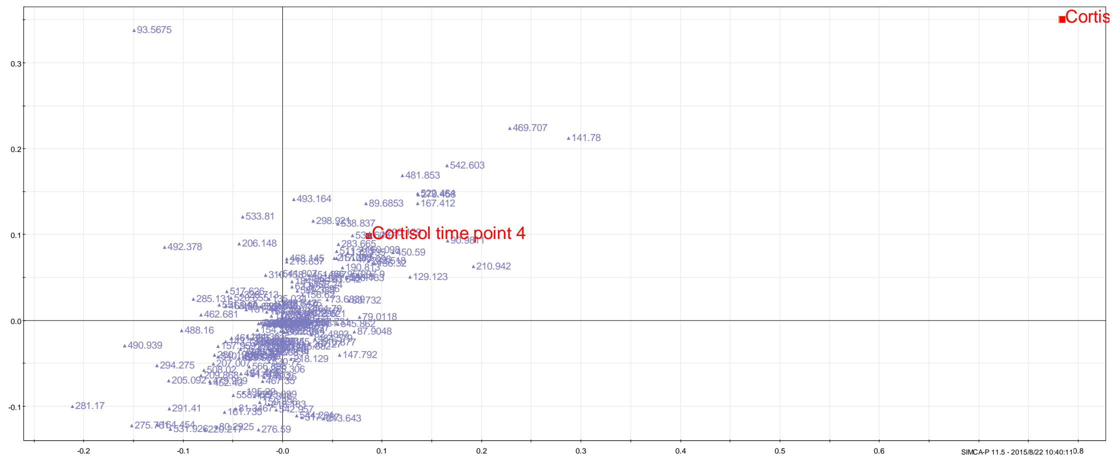


Figure 16a, b PLS Score and loading plot based on T-RFLP matrix *vs.* Max core temperature. Red box: Cortisol level at time point 2 (right) and 4 (left).

The subjects separated clearly in several groups representing three treatments of same subjects (Fig. 16a). As for the loading plot, a shift in microbiota can be seen from Fig. 16b. Moreover, Cortisol level at time point 4 had a bigger influence on the shifting.

DISCUSSION

The principal aims of the present study were to examine the latent heat stress relieving capacity of drinking buttermilk and to observe the human gut microbiota in hot occupational condition. Although no significant differences in diversity indices of three treatment groups were found, we detected, by means of salivary cortisol level analysis, significantly differences between the Buttermilk and Dehydrated groups, as well as Water and Dehydrated groups at the last time-point (P = 0.017). Additionally, the saliva cortisol level of subjects drinking buttermilk and water were all within the normal range (Kirschbaum *et al.,* 1994 and Kirschbaum *et al*., 2000), while at the last time point the cortisol level of the dehydrated group was above the normal salivary cortisol level. An increase in cortisol is usually thought to be indicative of stress (Evans *et al.,* 1994). However, there are three identified main characteristics of a stressor that determine the stress response, i.e., a stressor must be perceived as a new, and/or unexpected, and/or uncontrollable by the person (Mason, 1968). In this case, the experiment setting of stressor might not conform to these three characteristics. Thus, it could be a reason that the salivary cortisol profiles did not differ much among three treatments.

When it comes to the qPCR data, *Lactobacillus* and *Enterobacteriaceae* were detected in 9 (75%) and 12 (100%) subjects, with the medians of 4.039 (Buttermilk), 4.02 (Dehydrated), 4.081 (Water) and 7.407 (Buttermilk), 6.763 (Dehydrated), 7.64 (Water) log 16S rRNA gene copies/g rectal samples respectively. No significant difference in *Lactobacillus* or *Enterobacteriaceae* load of three treatments could be found. Due to limited time and project budget, only two bacterial groups were tested with qPCR analysis. However, higher IQR vales were found in *Enterobacteriaceae* population, which may indicate the treatments had larger influence on the population of *Enterobacteriaceae* group. From the correlation and linear regression test, no relation between the cortisol profile and *Enterobacteriaceae* population was found.

The PCA score and scatter plots indicated that the microbiota differed widely between individuals with regard to both composition and diversity. While the PLS score and loading scatter plots based on cortisol, core temperature and T-RFLP data explicated that the observations separated in groups which representing three treatments of same subject. However, the relationship between the stress indicators (core temperature and salivary cortisol level) and the gut microbial diversity was not clearly shown. As the PCA

and PLS models shown, the results clustered together mostly according subjects, which may indicate the gut microbiota itself differs from man to man. Inheritance and diet are in the long run the two most important factors for influencing the composition the gut microbiota of an individual. When the bacterial composition of 106 faecal samples, from 60 different mammalians (including humans) was compared, it was concluded that both the diet and phylogeny influenced the bacterial diversity (Ley et al. 2008).

It also should be stressed that, the order of three treatments were randomly decided. Thus, it might be difficult to observe the microbiota diversity, as well as the cortisol level differences, if buttermilk has certain effects on soothing the heat stress condition. Moreover, due to the subjects had different schedules, the second or third test of some subjects took several months after the first test was done. Hardly surprisingly, diet was reported to be the key spark for the development of intestinal microbiota structure (Yatsunenko T *et al.,* 2012). Researchers have found that long-term dietary patterns largely determine the main phyla of the gut microbial profile (Moschen AR *et al.*, 2012). On the other hand, administration of singular or small groups of select beneficial microbes although may not have a major impact on stable phyla, for example, a single strain of *Lactobacillus*, one that might be carried with traditional foods, may improve overall microbial diversity (Karlsson C *et al.*, 2010). The administration of a single *Bifidobacterium* strain, one among a genera commonly found in fermented dairy products, can increase the intestinal quantity of completely separate *Bifidobacterium* species, and Lactobacilli overall (Lahtinen SJ *et al.*, 2009; Ahmed M *et al.,* 2007). Thus, in this study, dietary pattern was an important factor that may affect the diversity indices.

Likewise, when it comes to cortisol profile, time after wakening is an important factor that may gave a different test result. Generally, the peak concentration is reached within 45 min in healthy subjects who wake up early (from 4:55 to 8:03 UT) and within 30 min in subjects who wake up later (from 8:24 to 12:03 UT) (Udielka and Kirschbaum, 2003). Then the cortisol concentration starts to decrease, decreasing slower in those who woke up early (Edwards S. *et al*., 2001). Although in this study, most of the subjects had the first salivary cortisol test at least one hour after they woke up (except one subject, S6, which was 40 minutes after waking), they still had different waking time that may have an influence of their cortisol profile, not to mention some of the subjects woke up very early to make it on time, which might stress the subject and give another cortisol profile.

Overall, the study although did not give significant results of most analyses with exception of the last time point salivary cortisol level data. However, from this study we gained a lot of practical experiences about dealing with human rectal and saliva samples in order to get an overview of the relation between gut microbiota and cortisol profile. In one word, it was a very complex study since a lot of environmental factors and personal factors need to be considered, on the other hand, the study gave us a lot of practical experience and thinking taking account of future perspectives.

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Appendix

Appendix 1 Individual qPCR results

Table 1A Log 16S rRNA gene copies /g rectal samples of different subjects

	Test person	log 16S rRNA gene copies/g rectal samples		
		Buttermilk	**Water**	**Dehydrated**
Lactobacillus	2	3.940	4.145	4.285
	3	4.086	4.122	4.076
	4	4.039	4.026	4.081
	6	4.003	3.772	3.914
	7	4.311	*	3.943
	9	3.953	*	4.197
	10	4.109	4.040	3.831
	11	4.044	*	3.994
	13	4.120	4.209	4.037
	15	3.937	4.125	4.020
	16	3.972	3.982	4.020
Enterobacteriaceae	2	3.313	8.123	7.216
	3	8.693	9.113	6.580
	4	8.278	8.823	9.330
	6	7.380	7.868	6.312
	7	8.420	8.049	3.517
	9	8.073	7.215	8.228
	10	4.195	4.742	7.945
	11	3.830	3.508	3.465
	13	7.434	7.412	6.946
	15	5.063	5.725	3.578
	16	4.854	7.073	8.573

* Under detection limit: 10^4 genes/reaction for the *Lactobacillus* and 10^2 genes/reaction for *Enterobacteriaceae*

Appendix 2 Buttermilk ingredients

Ingredients

➢ Thick curd/ plain yogurt	1 cup
➢ Water*	4 cups
➢ Green chilli	2 nos
➢ Chopped ginger	1 tbsp
➢ Chopped coriander	1 tbsp
➢ Chopped curry leaves	1 sprig
➢ Salt	As needed
➢ Asafoetida	1 pinch
➢ Lemon (optional)	1/2 tsp

(*Adjust water quantity depending upon curd's thickness and sourness.)

To Temper:

➢ Oil	1 tsp
➢ Mustard	1 tsp
➢ Asafoetida	1 pinch
➢ Curry leaves	1 sprig

Method

Step 1: Grind Green Chillies and Ginger

Step 2: Chop Coriander, Curry leaves finely

Step 3: Temper Oil, Mustard, Asafoetida, Curry leaves

Step 4: Beat the curd and add all the above

Step 5: Add water, salt and dilute well. If needed squeeze the lemon

Step 6: Make sure you beat/ whisk the buttermilk well, the frothy top adds a lot of flavor