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Full Length Research Paper

Effect of blueberry on the bacteria flora of the gut with special reference to *Lactobacillus*

Badina Abdulrasak Ige

Food Hygiene, Division of Food Technology, Department of Food Technology, Engineering and Nutrition, Lund University, Lund, Sweden.

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Blueberry (*Vaccinium species*) is an example of a fruit with high content of anti oxidative poly-phenols, and it has during the recent years been much noticed by its surprisingly potent effects against different diseases. Furthermore it can act as an antimicrobial agent in the gastro-intestine. This work looks into the effect of blueberry on the lactobacilli in the gut. *Lactobacillus* were isolated from feacal samples and identified to the species level. *Lactobacillus* were identified by subjecting to polymerase chain reaction (PCR) based randomly amplified polymorphic DNA (RAPD) for grouping. Representative isolate were then identified to specific species level by 16s-rRNA gene (rDNA) sequencing and to identify in comparison of the sequences with the GenBank data up to specific species level. The result shows that various *Lactobacillus* species were found with *Lactobacillus plantarum* having dominating the gut of one or two of the subject throughout the experiment. Some of the initial *Lactobacillus* found in the gut of subjects (for example *Lactobacillus acidophilus*) was during the administration period turning into *L. plantarum*. The changes that sometimes occur in the gut environment system also rears it head when one the voluntaries start with a growth of *Lactobacillus salivarius* and end up with strains of *Pedicoccus pentosaceus* and *Bifidobacterium longum.*

Key words: Blueberry, polymerase chain reaction (PCR), randomly amplified polymorphic DNA (RAPD), bacteria, deoxyribonucleic acid (DNA), *Lactobacillus species*, *Enterobacteriaceae.*

INTRODUCTION

The consumption of edible plants, fruits and vegetable has been demonstrated to prevent the occurrence of a number of diseases in humans and animals (Osman, 2006), example cancer, heart, vascular diseases and neurodegenerative diseases that all are associated with oxidative damage to lipids, proteins and nucleic acids by free radicals (Prior et al., 1998). Vegetables, fruits and their seeds are rich sources of vitamins C and E, beta-carotene and protease inhibitors, compounds that might protect the microorganism against free radical-induced injury and diseases (Osman, 2006).

Blueberry (*Vaccinium species*) is an example of a fruit with high content of antioxidative poly-phenoles, and is has during the recent years been much noticed by its surprisingly potent effects against different diseases (Osman, 2006). In the present study, RAPD analysis was performed in order to identify, at least in the case of lactobacilli, at the subspecies level and 16s-rDNA sequencing identifies at the species level and above.

The intention with the study is to clarify to what degree a consumption of blueberry can affect the composition of the human gut flora in respect of the load of *Lactobacillus*, and *Enterobacteriaceae*. The dominating strains of *Lactobacillus* were identified to the species level. The nutritional properties of blueberry as obtained from the United States department of Agriculture (USDA)

*Corresponding author. E-mail: rasaqbadina@yahoo.co.uk. Tel: +4746246756.

Table 1. Nutritional properties of blueberry

Principle	Nutrient value
Energy (kCal)	57
Carbohydrates (g)	14.49
Protein (g)	0.74
Total fat (g)	0.33
Cholesterol (mg)	0
Dietary fibre (g)	2.4
Vitamins	
Folates (µg)	6
Niacin (mg)	0.418
Pantothenic acid (mg)	0.124
Pyridoxine (mg)	0.052
Riboflavin (mg)	0.041
Vitamin A (IU)	54
Vitamin C (mg)	9,7
Vitamin E (mg)	0.57
Vitamin K (µg)	19.3
Electrolytes	
Sodium (mg)	1
Potassium (mg)	77
Minerals	
Calcium (mg)	6
Iron (mg)	0.28
Magnesium (mg)	6
Zinc (mg)	0.16
Phyto-nutrients	
Carotene-β (µg)	32
Lutein-Zeaxanthin (µg)	80

http://ndb.nal.usda.gov/, national nutrient database is shown in Table 1.

MATERIALS AND METHODS

Preparation of the juice

Blueberry fruits were obtained from a farm in Sweden and crushed into bulk puree and kept in the deep freezer (-20°C) (Sourced and processed by a company Probi AB, Lund, Sweden) which were dispensed into 300 ml clean plastic bottle with cover. They were then subjected to heat treatment in an autoclave at 100°C for 20 min in a batch process to reduce contaminating microorganisms. The heated buffers were put on ice for cooling.

The bottles were distributed to four volunteers (females between the ages of 20-30years) and they were advised to keep some of them in the freezer to preserve them. The volunteers were asked to consume 600 ml per day for 29 days (4 weeks and 1 day).

Feacal sample preparation

The feacal samples of the voluntaries (taking from early morning defecates by volunteers and kept in a sealed sample bottles) were taken thrice: The first sample was taken before consumption of the blueberry. The second after two weeks of consumption and the third, was taken the last day of consumption. Each feacal sample were analyze at the time of delivery.

A small quantity of the feacal sample was taken and weighed into sterile dilution media of 9 ml (preparation in the index below). The samples were thoroughly shaken to dissolve completely in the media. One milliliter (1ml) of the sample from the original diluted media were then taking and mixed into another tube containing 9 ml of sterile dilution media. After shaking, another 1 ml was taken and mixed with another tube containing 9 ml of sterile dilution media and this were continued until the desired dilution had being obtained, in this case 10^{-6}. 0.1 ml sample from each dilution media were taking and spread on an agar plate containing VRBD agar to test for the *Enterobacteriaceae* and the Rogosa agar for the testing of *Lactobacillus*. These were shaken for proper spreading of the sample with sterile glass beads.

The agar plates were incubated anaerobically (for the *Lactobacillus spp*) and aerobically (for the *Enterobacteriaceae*) at 37°C for 72 and 24 h respectively. The plates were then counted and recorded based on colony forming unite per gram feaces (CFU/g).

Pure culture cultivation

The *Lactobacillus* colonies were randomly picked and re-cultured in a new rogosa agar plates for purity in the incubator at 37°C for 72 h. The pure cultures which were harvested into an Eppendorf tube containing (Osman, 2006) water media (autoclave milliQ water) and (Karakaya et al., 2001) Freezing media.

The bacteria in the water media were kept at -20°C for the RAPD and 16S-rDNA analysis whiles the one in the freezing media were subjected to -80°C to preserve them for further use.

Preparation of DNA for RAPD

The crude cell extract were prepared according to protocol described by Johansson et al. (1995) and Quednau (1998). Isolates were randomly picked from the countable plates and were centrifuged in an Eppendorf tubes and washed twice with 500 µl- 1 ml double distilled water. After washing, cells were resuspended in 250-500 µl double distilled water. The cells were disintegrated by shaking together with 10-12 sterile glass beads (2mm in diameter) for 45 min at 4°C using an Eppendorf mixer. After centrifugation at 14,000 rpm for 5 min, 1 µl of supernatant was used for the PCR reaction.

The polymerase chain reaction of random fragments was run with 73 primer (5`-ACGCGCCCT-3`; Swedish Gene Synthesis, Koping, Sweden). Agarose gel electrophoresis was run: The gels were stained with ethidium bromide and photographed under UV illumination.

Procedure for 16s-rDNA PCR

The procedures were done as described by Wang (2004). Based on the cluster analysis by RAPD band patterns of all isolates, strains that represented tight clusters and isolates forming single number clusters and isolates forming single number clusters were selected for 16S rDNA sequencing.

The generated sequences were compared to the GenBank database (National Centre for Biotechnology Information, Rockville Pike, Bethesda, MD, USA). Isolates belonging to the same RAPD cluster as a sequenced isolate were judged as having the same species identity.

Table 2. Result showing bacteria counts of subjects.

Subject	CFU/G	LOG
A*	$4.53 * 10^7$	7.66
B*	$5.5 * 10^5$	5.74
C*	0	0
D*	$5.9 * 10^6$	6.77
A**	$5.3 * 10^7$	7.72
B**	$2.1 * 10^8$	8.32
C**	0	0
D**	$1.3 * 10^5$	5.11
A***	$1.9 * 10^7$	7.28
B***	$6.7 * 10^7$	7.83
C***	$1.6 * 10^4$	4.20
D***	$1.3 * 10^6$	6.11

Table showing growth on plates counted and recorded based on colony forming unit per gram (cfu/g). Where: A, B, C and D = subject (observations is stated after the tables); * = Feacal samples taking before commencement of experiment; ** = Feacal samples taking two weeks into the commencement of experiment; *** = Feacal sample taking four weeks into the commencement of experiment.

Table 3. Result showing bacteria counts of subjects

Subject	CFU/G	LOG
A*	$1.6 * 10^6$	6.20
B*	$2.1 * 10^5$	5.32
C*	$9.3 * 10^6$	6.97
D*	$5 * 10^6$	6.69
A**	$9.5 * 10^5$	5.98
B**	$1.5 * 10^7$	7.18
C**	$8.1 * 10^6$	6.91
D**	$1.1 * 10^8$	8.04
A***	$5.4 * 10^5$	5.73
B***	$5.4 * 10^5$	6.15
C***	$6.1 * 10^6$	6.79
D***	$4.2 * 10^7$	7.63

Table showing growth on plates counted and recorded based on colony forming unit per gram (cfu/g). Where: A, B, C AND D = subject (observations is stated after the tables);* = Feacal samples taking before commencement of experiment; ** = Feacal samples taking two weeks into the commencement of experiment;*** = Feacal sample taking four weeks into the commencement of experiment

RESULTS

Tables 2 and 3 show that a considerable reduction of the *Enterobacteriaceae* occurs in the weeks of consumption and also a little increase in *Lactobacilli* but these could not ascertain due to variations in some of the subjects microbial gut as presented in the colony growth result and the 16S-rDNA sequencing done on the BLAST species level of their feacal sample (Table 4). A previous studies by Osman (2006) and Prior et al. (1998) mentioned closely related result as above.

Figures 1 and 2 show the picture of the RAPD band patterns which shows the occurrence of the same band pattern showing bacteria of the same likely species and also it gives preliminary result of suspected strain. The result also visualized the changes in the bacteria species in this case lactobacilli; when lies at the same pattern in the band (Figures 1 and 2) as confirmed by the study of Power (1996) and Johansson et al. (1995). These were explained more in the discussion section.

Table 4 shows the most likely strains of the bacteria DNA sequenced using the 16s-rDNA sequencing. On comparing with the strains of bacteria on the GENBANK the result on the table above gave the likely result.

DISCUSSION

The result of the bacterial count (Tables 2 and 3) gave an insight into the hypothesis that the consumption of the blueberry tends to affect the number of *Enterobacteriaceae* with the number of *Lactobacillus* species. A previous study by Osman (2006) and also Prior et al. (1998) shows that blueberries have outstanding antioxidant capacity which is correlated with their Anthocyanins and total phenolic content and that this antioxidant capacity tends to acts as antimicrobial in the gut. These affect the balance in the gut ecosystem thereby reducing the numbers of some harmful bacteria in the gut and giving room to some that can resist the antioxidant capacity to grow (Karakaya et al., 2001; Prior et al., 1998). But in this present study this could not be concluded because of the small numbers of subject but gave an insight into the presence of these strains of bacteria in the gut and that they are responding to the consumption of the blueberry in a way. The consumption of the blueberry affects gut ecosystem through the emergence of new strain of lactobacilli and some other strain of bacteria which were not detected before the start of the consumption but which later was discovered to emerge in the gut of some of the volunteers.

Randomly amplify polymorphic DNA (RAPD) of the *Lactobacillus* isolate visualize the changes in the bacteria species from the gut. RAPD also reveals the close relative of some species when these lies at the same pattern in the band.

The present study shows the changes occurring in the emergence of different species of lactobacilli after the blueberry consumption (Figures 1 and 2). Past studies by Johansson et al. (1995) and Power (1996) confirms that these changes are identified through band patterns with different spaces and some lying in the same level. The RAPD-pattern (Figures 1 and 2) shows the differences

Table 4. Table showing the result of 16S-rDNA sequencing done on BLAST.

Volunteers (Week)	A	B	C	D
1	*Lactobacillus plantarum* (97%)	*Lactobacillus salivarius* (98%)	*Lactobacillus rhamnosus* (98%)	*Lactobacillus species*
2	*Lactobacillus plantarum* (100%)	*Lactobacillus salivarius* (96%)	*Lactobacillus rhamnosus (99%)* *Lactobacillus delbrueckii (100%)*	*Lactobacillus acidophilus* (100%)
3	*Lactobacillus plantarum* (99%)	*Pedicoccus pentosaceus* (100%) *Bifidobacterium longun* (98%)	*Lactobacillus paracasei* (100%) *Lactobacillus reuteri* (100%)	*Lactobacillus plantarum* (100%)

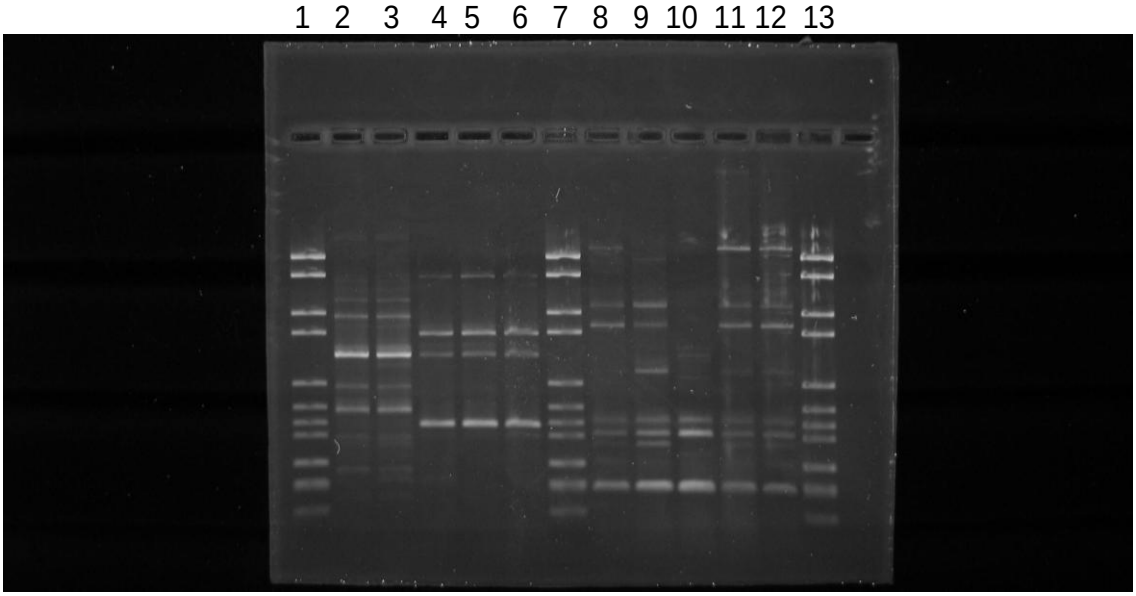

Figure 1. RAPD band patterns for different *Lactobacillus* strains. Lanes 1, 7 AND 13 are the DNA size markers. Lanes 2, 3, 4, 5, 6, 8, 9, 10, 11 and 12 represent different strains of Lactobacilli species: These include Lactobacillus plantarum (Band 2 and 3), Lactobacillus salivarius (Band 4, 5 and 6), *Lactobacillus rhamnosus* (Band 8 ,9 and 10), Lactobacillus acidophilus and *Lactobacillus paracasei* (Band 11 and 12).

between the strains of *L. plantarum, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus paracasei, Lactobacillus reuteri* and the emergence of *Pedicoccus pentosaceus* and occurrence of *Bifidobacterium longum* with completely different band pattern. The result in Table 4 reveals the dominancy of *L. plantarum* throughout the study in one of the volunteers with its band pattern being the same throughout and the result of the 16S rDNA confirmed it.

The 16S-rDNA sequencing and the identification of the sequence with the Genbank stored database reveals the strains developing through the study and also the dominance of some of the strain.

It has being reveal that the strain of *L. plantarum* resist the anti-oxidant capacity of the phenolic in the blueberry by remaining consistently in the gut throughout the experimental period and even increasing in numbers in one of the subject. Osman (2006) also confirms this in his study on combining blueberry with a strain of *L. plantarum* which improves the disease index of some intestinal bowel diseases. This anti-oxidant property of the phenol in the blueberry shows itself when it was revealed in one the volunteers that the strain discovered was *L. plantarum.* In one of the subject, *Lactobacillus salivarius* also prove to resist the anti-oxidant capacity of the blueberry but it sound interesting that at

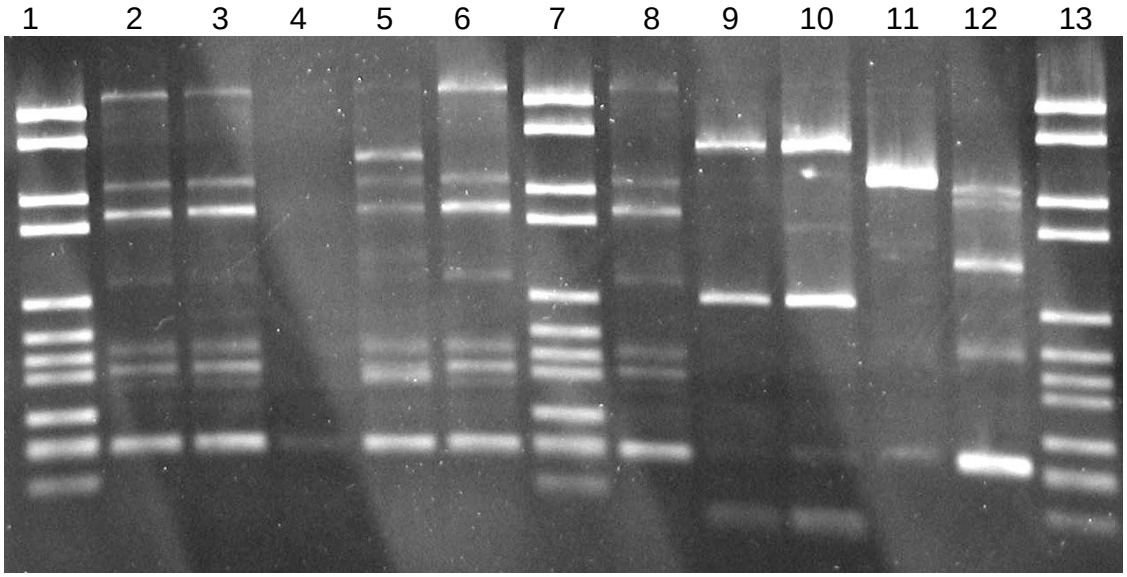

Figure 2. RAPD band patterns for different *Lactobacillus* strains. Lanes 1, 7 And 13 Represents Universal Primers. Lanes 2, 3, 4, 5, 6, 8, 9, 10, 11, 12 represent strains of *Lactobacilli* Species: These include *Lactobacillus plantarum* (Band 2 and 3), *Lactobacillus salivarius* (Band 4 and 5), *Lactobacillus rhamnosus* (Band 6*), Lactobacillus acidophilus* (Band 7, 8 and 9), *Lactobacillus paracasei* (Band 8 and 9), *Lactobacillus delbrueckii* (Band 9 and 10), *Pedicoccus pentosaceus* (Band 11) and *Bifidobacterium longum* (Band 12).

the latter end it gave way to the development other lactic acid bacteria of the strain but different morphology *P. pentosaceus* and *B. longum*. This also proves that consumption of blueberry could lead to the emergence of new strain of beneficial bacteria.

The changes in the bacteria species during consumption of the blueberry also reveals itself through the analysis of the sample from the other two volunteers who started with the development of *L. rhamnosus* and *acidophilus* but ended up with *L. paracasei, L. reuteri and L. plantarum*, respectively.

Above all, emergence of *L. plantarum* were consistent in all the subjects and prove itself to be a strain that occur frequently and this could be due to its resistance to the anti-oxidant properties of the blueberry. This may be due to its frequent and wide occurrence in nature and use as a starter culture in food and feed fermentation as stated by Johansson et al. (1995).

The use of RAPD and 16S-rDNA polymerase chain analysis as a means of identifying unknown strain of lactic acid bacteria to the species level proves to be effective. And the use of the nucleotide sequence data ensures that the results are highly reproducible and conclusive.

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