.

Microorganisms' identification by Colony – Polymerase chain Reaction and Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry Biotyper (Bruker)

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Abstract

Bifidobacteria Identification is important in medical and pharmaceutical industry. Molecular analytic technique such as PCR gel electrophoresis and MALDI –TOF-MS are high sensitive and useful technique in identifying Bifidobacteria. This practical was planed to identification of Bifidobacteria by both method.

PCR method is based on denaturing, annealing and extension of target DNA in the bacteria in vitro with other requirements like taq polymerase enzyme, DNA primers, Mg etc with temperature control. MALDI –TOF-MS is based on analyet Matrix co crystallization and firing of laser beam get ionization and detection time of flight by mass spectrometry

Un known 3 cutler plats (S1, S2,S3) were used for both methods and correctly identified up to genus level by PCR agarose gel electrophoresis. MALDI TOF MS was done in direct application and formic acid extraction method. Both ways identified up to bifid bacteria species and lactobacillus. Both methods were equally highly sensitive to identify Bifidobacteria.

Identification of species was highly accurate MALDY TOF –MS method. But both methods have done with limited sample size. Both method are complex, high cost and need high technical knowledge. MALDI method results depend on software apart from other steps.

PCR can improve to identify Bifid bacteria up to species level with use of species level primers .MALDI method with formic acid exaction can improve sensitivity with purification of protein.

(PCR-polymerase Chain Reaction .MALDI-TOF-MS- Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry).

1. Introduction.

Identification of microorganisms is the key factor in the diagnosis, management of communicable disease .Also it is essentials in environmental and industrial development providing safe food and nutrition in all over the world.

Microorganisms' identification method varies from morphological light microcopy to electron microscopy, or cutler and microscopic colony identification and gene or protein detection by PCR or MALDY –TOF and deferent serological methods. .(Silvan et al 2011). .(Silvan et al 2011).

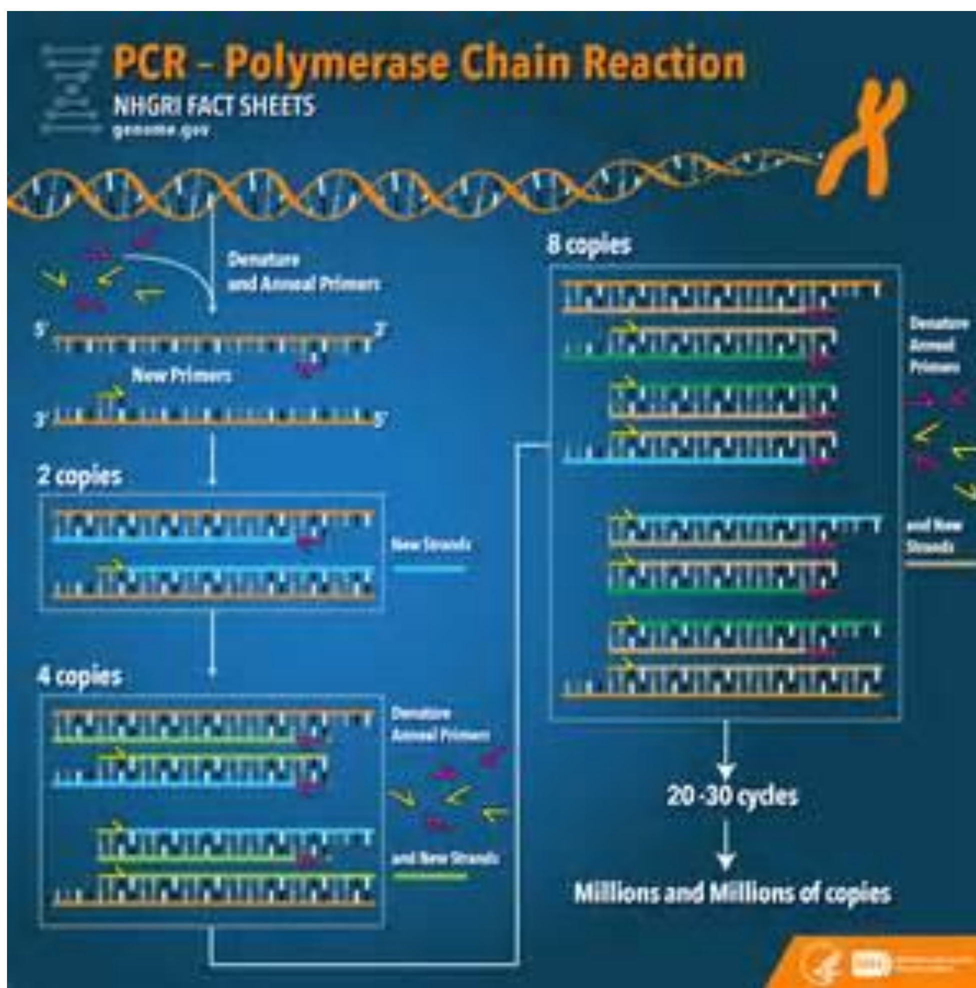
Bifidobacteria is gram positive, pleomorphic, anaerobic, and non-motile rods found in human gastro intestinal tract (commensal flora.) It has identified 32 species. Identification of species is difficult by appearance of light microscopy or culture colony.(Hong and Chen 2007)

Identifying of bacteria by method of PCR and MALDY –TOF-MS practiced due to high sensitivity and specificity. Therefore this practice was planned to do with both methods. Rationale of the use of both tests is to be discussed.

Polymerase chain reaction is DNA or RNA amplification test by using single or fragment of DNA/RNA in vitero.(similar to in vivo).DNA amplification is a 3steps process, such as denaturation, annealing and extension. Denaturing is done by heating and double standard DNA separation into single stand. Then annealing starts and the Primer will hybrid with single standard DNA while the temperature is being lowered. After that new double standard DNA undergoes extension with optimum temperature. Only repeating of temperature changing cycles multiple DNA template were produced with presence of primer ,heat stable DNA polymerase enzyme ,Mg++ and buffer. .(Silvan et al 2011). .

Next step is separation of DNA molecule by Gel electrophoresis. Negatively charged DNA molecules move towards the cathode (+ charge) in the electric field. The rate of migration of molecule depends on sample, Electric field, medium and buffer. When considered sample's high charge/mass ratio (inversely proportionate to molecular weight), low size, shape (globular) give rise to fast separation. High potential gradient give fast mobility. If the Medium has more adhering tendency efficiency will be diminish. Apart from that cross link structures make pores and the small molecule passes easily through pores. Super coil DNA moves faster due to their shape. But liner and circular DNA molecule tent gets slow in the field. Buffers with low ionic strength separation will be fast. If the PH is high, increase in ionization (Organic acid) or low PH increases ionization of organic base and separation will be low. Concentration of Agarose gel facilitates separation of small DNA fragment. Lager DNA molecule separation is fast in low concentration. Large DNA molecule migrates fast in high voltage in the electric field and the small DNA molecule needs low voltage to be fast. If DNA binds to Ethylene bromide the molecular migration is slow. .(Silvan et al 2011).(Jaksan et al 2014).

Finally Separated DNA molecule can be detected under UV light for interpretation. DNA molecule binds with fluorescent dye and it appears in green colure under UV light. Band containing 20ng of DNA is visualized under the UV light transilluminator. (Bushell and Burns 2012)



Figuer-1 DNA amplification in PCR, Denaturing, annealing and extension steps. (Obtained from http://www.medscape.org).

MALDI –TOF –MS detection technique is based on identification of bacteria (bacterial protein) by the use of laser radiation to get ionized analyte within the matrix and mas spectrometric detection. It has four main steps such as sample preparation, Ionization, detection and data analysis. (Biswos and Rolah 2012)(Barberies et al 2010).

MALDI gives nondestructive vaporization and ionization of molecules. Analyte is co-crystalized with matrix compound. Therefore, laser radiation vaporizes matrix (Analyate within the matrix) and has the property of proton donor or receptor. analyte Ionization depends on ionic nature of matrix –analyte combination. All ion are getting same amount of energy but they are coming to detector at deferent time. Time of Flight depends on mass/charge (ratio) and kinetic energy of ion. MALDI combined with TOF limit of resolution (m/z) is more than 300000. Bacteria from colony or extracted bacterial protein is placed as target sample and overlaid matrix. After that it is allowed co crystallization. Laser irradiation should be done. Mass spectrum will be analyzed by software and compared with stored data (data finger print) of bacteria to identify Bifidobacteria. .(Hong and Chen 2007) (Biswos and Rolah 2012)

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AIMS of the Practical.

1. Identify bacteria in clinical specimen.

Identify bacterial species (gram negative) in culture (colony) DNA amplification (PCR) and detection protein (MALDI –TOF –MS). Suspected sample of Bifidobacteria identification and confirmation.

2. Compare the sensitivity of PCR and MALDI-TOP-MS to detect bacteria.

Extent of identify bacteria (true positive) by both method.

3. Compare sensitivity the extended direct inoculation and formic acid extraction method in MALDI TOF MS.

4. Compare other factors (cost, complexity time instruments, manpower) of both method

5. Discuss the clinical uses in both method.

.**Method**

There are two methods

Method 1-PCR

Method 2-MALDI –TOF-MS

Method- 1.

PCR test was planned to do in 3 steps such as amplification (step –I) Gell Electrophoresis (step –II) and detection via UV light (Step –III). **BMS4977 Middlesex University** guidelines were followed.

Step –I **Polymerase Chain Reaction**

Step I was based on amplification of target bacterial DNA. Reaction occurred with Pair of primers (forward, revers), 4 types of Deoxyribose nucleoside Triphosphate (d NTP) Mg2 + and heat stable DNA polymerase Enzyme.

PCR master mix

Fresh 1.5ml Eppendorf tube was labeled “MM” and PCR mater mix was made in the tube according to following composition.

Table -1 component master of Mix

Components of PCR Master mix	Volume (need for one reaction)	Final concentration	Reaction Mix
2XPCR reaction Mix	12.5µl	1	62.5µl
10Mm Primer Mix(F+R)	2µl	1µM each	10µl
Sterile water	10.5µl		52.5µl
Total volume			125µl

PCR reaction Mix contained -Taq polymerase, buffer,$MgCl_2$, and dNTPs.

Primer Mix was contained –genus Bifid 1forword primer 5' to 3' (CTCCTGGAAACGGGTGG) and Bifid 2 reverse 5' to 3'(GGTGTTCTTCCCGATACTACA).

Procedure - PCR Tube.

Then MM tube has well mixed content of master mix. Four PCR tubes were labeled as S1,,S2,.S3 and C. Master Mix(25µl) was added in to each tube. Small amount of bacterial colony was added in to S1,S2, and S3 tubes. Small amount of colonies were taken from colony plate S1,S2 and S3 and added accordingly. Each tube was mixed well using sterile pipette tip. Control (C,) tube was kept without colony.

Procedure –Thermalcycler.

All 4 PCR tubes were placed in thermacycler. Programed thermalcycler was used. Tubes were kept until completion of automated processes. It has been programmed to heat 95 ^{0}C for 5 minute for denaturation of DNA initially. After that 95^{0}C for 30 seconds (denaturation) leads to 55^{0}C for 30 seconds (primer annealing) and at the end 72^{0}C 30th second. These cycles ran 3o times. Finally it was kept at 72^{0}C for 5 minutes. (Final extension). It was Chilled to 4 ^{0}C.

Step -II Separation **of DNA Fragment by Agarose Gel Electrophoresis**

Procedure-Electrophoresis tray was set in the tank and filled with Liquid Agarose. Eight sample comb was placed on side of the tray. It was kept in 15 minute to set the gel and comb was removed. (Tray was turned in to correct position) Then electrophoresis buffer was added into the tray until it covers the upper suffers of the gel. Then 5µl of loading dye was added into S1, S2, and S3.and control PCR tube. DNA ladder (8µl) was added in to 1st well and 25µl from each sample of S1, S2, S3, and C were added to the rest of the wells accordingly. System was connected to the power supply (75V). It was kept 40 minute until the complete ~~the~~ movement of dye and power was disconnected.

[1 % Agarose provided (dissolved in electrophoresis buffer -TAE) after boiling and cooled up to 60^0 C. Already Non carcinogenic Fluorescent dye was added to DNA stain.]

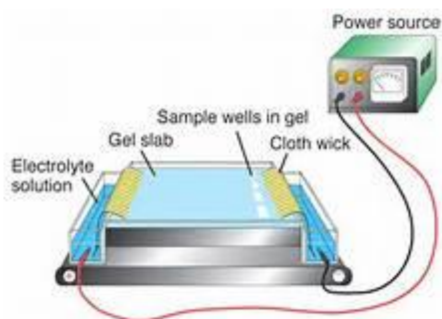


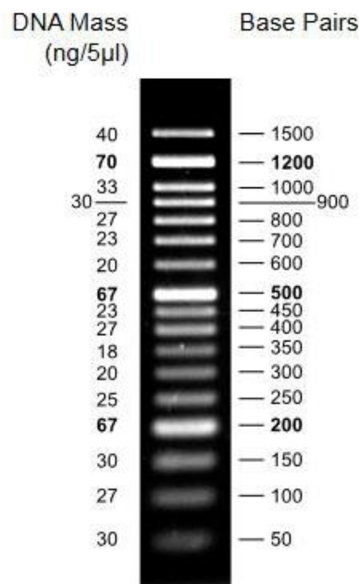
Image-1 Gel Electrophoresis chamber.(obtained from http://www.medscape.org)

Step -III

DNA- molecule detection under the UV light.

Procedure-Chamber of tray with gel was removed and inserted in to Spectrometer. It was inspected under the UV light and Photograph of DNA band was taken.

Identification of DNA base pairs were done in the result with comparison of slandered reference ladder given bellow.



2 % TAE agarose gel

- PCRBIO Ladder III

Image- 2 Standard ladder in 2% Agarose gel.

Method -2(fallowed guide- BMS4977 Middlesex University)

MALDI-TOF-MS.

According to the BMS biotyper protocol and Bacterial test Standard, (BTS Bruker) this practical was performed with already prepared and paced on laser irradiation MALDI plate. (Inoculated with same S1,S2 ,S3 colony culture plate with direct extended inoculation and protein extraction from bacteria).

Also calibrated Machine was used (before the practical according to protocol). Laser beam (energy) was fired on each S1, S2,S3, extended direct transfer and Formic acid extracted samples. Laser beams were spotted (fired) in different positions under the observation through the camera. Data were analyzed by software according to following steps .Flex analysis software was opened ,external calibration applied, then bio tool software was opened ,peptide mass fingerprint icon opened , after that setting selected .Then Biotyper Score (Score between 2.3-3) and with a description of bacteria genus and species were obtained.

Sample preparation and machine calibration (**Already performed before the practical according to Bruker database NCBI sequences /Taxonomy)**

Bacterial cultures were prepared to use for both practical. Colombia –Blood agar or Chocolate agar was used to prepare sterile culture plate and target clinical samples were inoculated into the plate and allowed to grow in room temperature.

Extended direct transfer sample was prepared with standard HCCA solvent 250µl in HCCA tube and dissolved recommended Matrix HCCA by vortexing at room temperature until clearance. Bacterial colony spotted on to MALDI target plate. 1µ Formic acid (70%) overlaid over the spotted colony on the plate and allowed to dry. Then 1µl of HCCA solution was overlaid the material allowed to dry in room temperature.

Formic acid extraction method was performed same bacterial culture colony (5-10mg /colony) mixed (vortexing) with 300µl of deionized water into the Eppendorf tube. Then 900µl EtOH was added and mixed. Mixture was centrifuged (13000-1500rpm) for 2 minutes. Supernatant was decanted and centrifuged, then residual EtOH was removed by pipetting. EtOH pellet was kept in room temperature to dry.(2-3 minute). After that 70% formic acid was mixed with pellet by vortexing. Same volume of CAN(1-80µl) was added centrifuged (max speed). The 1µl of supernatant was pipetted on to MALDY plate and allowed drying in the room temperature. HCCA matrix (standard)solution was overlaid spot of target (within 1 hour) and allowed air dried.

Calibration of machine software was adjusted (BTS Bruker part no #255343) to one target position.(overlay matrix solution)MBT_FC par flex control method was opened. Six to forty time laser shots were added from different positions and spectrum were measured and recorded .Energy levels were adjusted (Auto x method).

Calibration button was chosen and the calibration file was selected (MBT standard), correct calibration peaks were selected by pressing automatic Assign button.(maximum deviation up to 300ppm). New calibration was saved. Result were taken sample quality, best scour value with comparison colure cord given bellow by software. (Score values based on pattern matching algorithm of peaks in reference data and unknown data then convert in to log value 0-3).

Meaning of Score Values

Range	Description	Symbols	Color
2.300 ... 3.000	**highly probable species identification**	**(+++)**	green
2.000 ... 2.299	**secure genus identification, probable species identification**	**(++)**	green
1.700 ... 1.999	**probable genus identification**	**(+)**	yellow
0.000 ... 1.699	**not reliable identification**	**(-)**	red

Meaning of Consistency Categories (A - C) and Colour cord

1 A- Species consistency -Green-Identified species ,Yellow-Match with genus

2.B-Genus consistency- Green-Match with Genus,Yellow- Probably same Genus

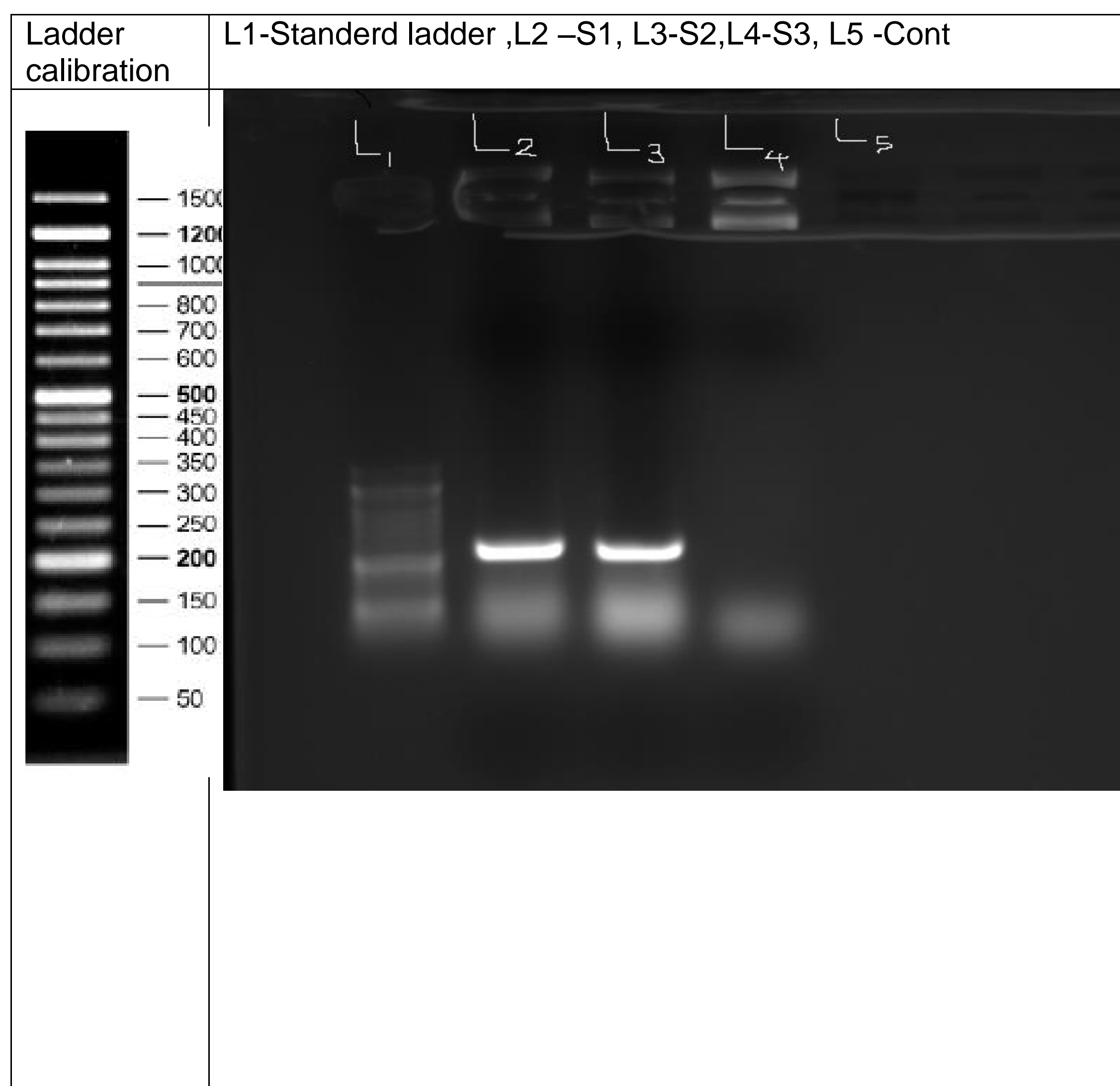
3.C-No consistency-Red-No match.

.Results

Both methods have given qualitative and quantitative data to identify the bacteria.

PCR test DNA fragment photo was taken under UV light (after electrophoresis) Transiluminator. Calculations of base pair logarithmic was used Microsoft Word excel software.

Image-2

Ladder calibration	L1-Standerd ladder ,L2 –S1, L3-S2,L4-S3, L5 -Cont
	

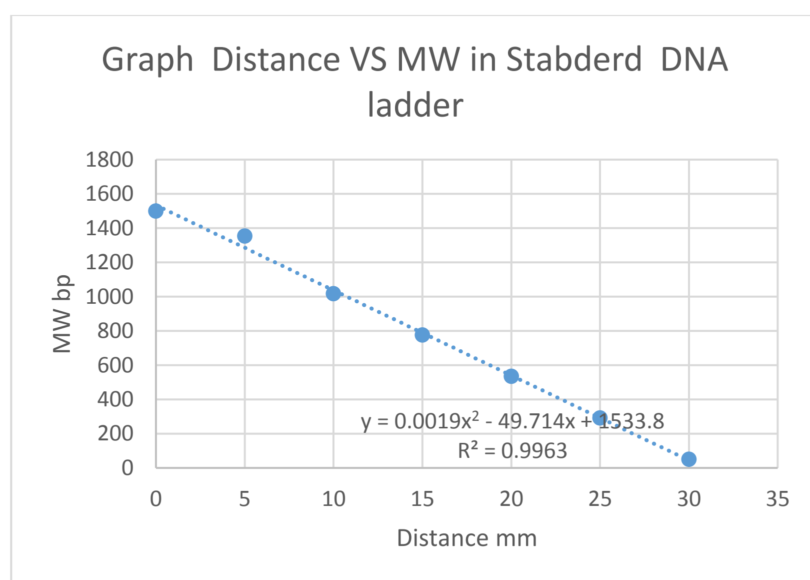
L=lain /L1-Standed ladder ,L2-S1,L3-S2.L4-S3,L5-Controle.

Image 3 – Imagers from left to right –DNA ladder(Lain 1), DNA fragment S1(Lain 2) ,S2(Lain 3),S3(Lain 4) respectively.S1 and S2 has clear DNA fragment closure cathode (+) end compatible 260bp .S3 has given fragmentation within the well compatible 1300bp. Control sample (Lain 5) do not visualized as bands .

Table -2

Standard DNA ladder distance and (molecular weight) MW in base pair

Distance -mm	DNA fragment bp
0	1500
5	1259
10	1017
15	775
20	534
25	293
30	50



S1,S2 =24mm(X) correspond to ______260bp(Y)

Graph -1 DNA ladder fragmentation in electrophoresis. Highest molecular weight of DNA fragment 1500bp moved nearly 0 distance mm and lowest molecular weight 50bp moved up to 30mm. S1 has given distance 24 mm compatible with 260bp and S2 also given 24mm compatible with 260bp and S3 4mm compatible with 1250bp

MALDI TOF-MS direct spotting method- 3 sets of samples score value results with best match of organisms were taken from S1, S2, and S3 target plate organisms. According to best rank quality high scours valve of 3 samples given separately.

Table -3 Summary of Best scour value in Extended direct Application.

AnalyteName	Organism(best match)	ScoreValue	Organism(second best match)	ScoreValue
Sample 1(+) (B)	Bifidobacterium breve	1.98	Bifidobacterium breve	1.791
Sample 2(++) (A)	Bifidobacterium longum	2.166	Bifidobacterium longum	2.15
Sample 3(+++) (A)	Lactobacillus paracasei	2.4	Lactobacillus paracasei	2.27

Sample 1 best scour values with highest quality (+) were 1.98 and 1.79. This valve is compatible to genus identification level of Bifidobacterium with comparison scores (Yellow). S2 highest quality (++) values were given score 2.166 and 2.15 compatible with confirmation of genus identification with probably species is Bifidobacterium longum.S3 was given highest quality (+++) with score valve 2.4 and 2.27 compatible with high probable species of Lactobacilus paracasei .

Result of Formic acid extraction method - S1, S2, and S3 were tested in two times each and summary of best of results noted.

Table -3, Summary of Best scour value in formic acid extraction method.

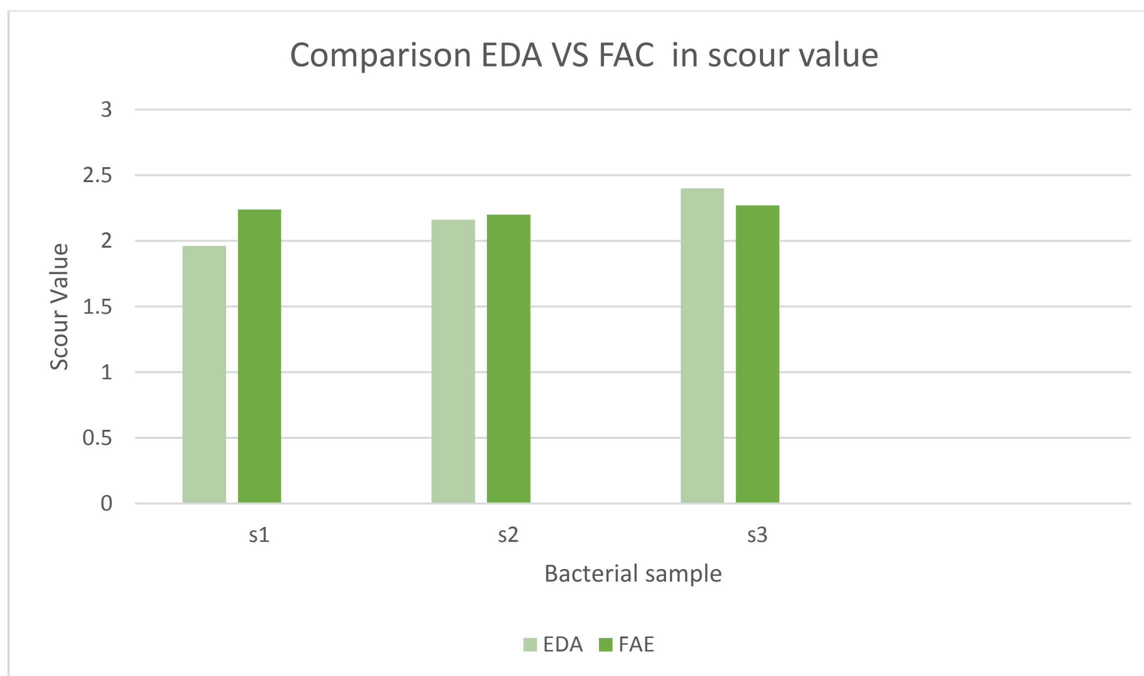
AnalyteName	AnalyteID	Organism(best match)	ScoreValue	Organism(second best match)	ScoreValue
H12(++) (A)	S1	Bifidobacterium breve	2.241	Bifidobacterium breve	2.12
H13(++) (A)	S1	Bifidobacterium breve	2.151	Bifidobacterium breve	2.11
H14(++) (A)	S2	Bifidobacterium longum	2.205	Bifidobacterium longum	2.139
H15(++) (A)	S2	Bifidobacterium longum	2.113	Bifidobacterium longum	2.093
H16(++) (A)	S3	Lactobacillus paracasei	2.059	Lactobacillus paracasei	2.054

H17(++) (A)	S3	Lactobacillus paracasei	2.25	Lactobacillus paracasei	2.228
J10(++) (B)	BTS	Escherichia coli	2.276	Escherichia coli	2.225

S1 highest quality (++) with high score 2.24 and 2.15 compatible with genus level confirmation with highest probability of species (green) Bifidobacterium brave. S2 given highest scour 2.205 and 2.139 with comparison highest possibility Bifidobacterium longum.S3 has given highest score 2.25 and 2.22 compatible with probably species level of Lactobacillus paracasei.

Table -4 Comparison of highest score valve in EDA and FAE MALDI method

MALDI method	Bifidobacterium brave	Bifidobacterium longum	Lactobacius paracasei
EDI	1.96	2.16	2.4
FAE	2.24	2.2	2.27



Graph – 2Comparison of Extended direct inoculation method with Formic acid extraction method in Bb(S1),Bl(S2), Lp(S3) bacteria. Extended direct inoculation has low valve in S1 and other two similar value

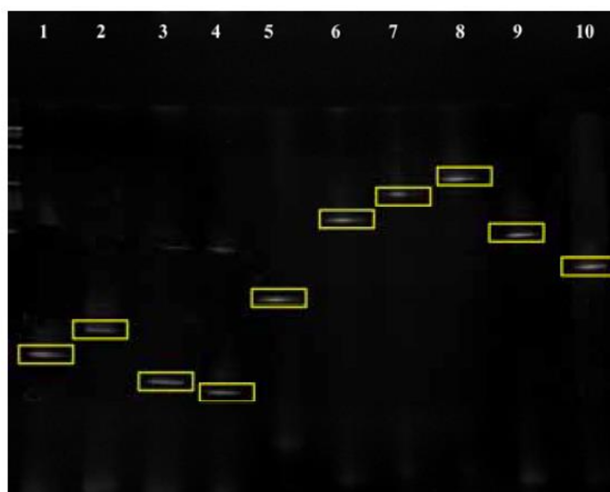
Discussion

Ideal bacterial identification system should be quick, accurate, simple, and reproducible. Also it should has capacity to minimized adverse cutler growing conditions.

Bifidobacteria was identified in this practical (Genus level) by PCR gel electrophoresis method comparison of base pair in S1 and S2 plate , also it has identified species level by MALDI TOF MS mthods.S3 plate bacteria was identified genus level by PCR gel electrophoresis method as Lactobacillus and Identified species of lactobacillus by MALDY TOF MS method.

PCR gel electrophoresis method has detected base pair between 200 to 300 is compatible with Bifidobacreia in (2%) Agarose gel electrophoresis. But both S1 and S2 were given similar level bands (same M/W) possibility of deferent species or same species of Bifidobaceria or almost similar sub species or gene mutation or deletion of Bacteria. There may be a possibility of human error such as introduction of same culture plate for both S1,S2 sample or same bacteria inoculate to both cutler plate Hong and chen 2007 were carried out similar experiment among deferent species of Bifidobacteria. They described B.longum and B.breve have not given similar band (figure..) pattern in gel electrophoresis. Therefore, without further evaluation can't be commented about species level.

Hong and Chen (2007) Asian-Aust. J. Anim. Sci. 20(12):1887-1894 **1891**



1. DGGE profiles of the PCR products originating from the reference strains used in this study. Lane 1: *B. angulatum*; Lane 2: *B.* *r*; Lane 3: *B. longum*; Lane 4: *B. infantis*; Lane 5: *B. animalis*; Lane 6: *B. breve*; Lane 7: *B. catenulatum*; Lane 8:*B. minimum*; : *B. subtile*; Lane 10: *B. thermophilum*; Lane 11: mix samples.

Image -4

Also Collado undergone study of Lactobacillus and successfully amplified ribosomal DNA restriction analysis of Lacto bacillus and Bifidobacteria in diary product up to species level. (Collado and Nadez 2007).
Bushell, and Burns,(2012) were studied identification of probiotic bacteria in species level by PCR match with sequence database .They found low sensitivity due to contamination.
MALDI TOF MS method given higher scour valve for B.brave in formic acid extraction method than direct application. Both application has identified B.breve is the most possibility in S1 plate. Direct application method has given low scour may be due to contamination. Identification of S2 plate both (direct/formic acid) given similar scour valve and species as B.longam. S3 plate has given high scour valve for direct method and it has confirmed as it is Lactobacillus parsai.

Accuracy sensitivity and specificity to be discussed in detail of PCR gel Electrophoresis and MALDI –TOF-MS method. PCR gel electrophoresis has identified S1 andS2 as same bacteria (Genus level) but MALDI TOF MS identified it as deferent species in S1 and S2 plate. Therefore Identification up to species level MALDI TOF MS is more sensitive than PCR. But PCR also able to identify species level correctly if further analysis carried out, such as species level primer should be used (Genus level primers were used for this practical). When considered sensitivity and specificity of both method PCR method has high sensitivity and specificity to detect bacteria at Genus level but MALDI –TOF-MS has very high sensitivity up to species level .This practical only single PCR test was done if multiple attempt were made sensitivity and specificity could be calculated. Koskinan et al in 2008 studied bovine mastitis 9 pathogens (sample size n=407), identified by PCR and 100% sensitivity and specificity at genus level. MALDI –TOF MS method has identify correctly up to species level in this practical in all attempts. But number of sample is minimum and each sample tested two times. Canadian agency for drug and technologist publish MALDI—TOF. MS sensitivity and specificity up to the species level for several human pathogenic bacteria about 80-99% range. Vermeersch et al 2007undergone experiment to compare sensitivity of both method by identifying SRY gene in fetus. They conclude MALDI TOF MS has 98% and PCR has 96% sensitivity. Barberies and his team were carried out study to identify human pathogens by MALDI –TOF-MS and conventional PCR sensitivity genus and species level 93,52% vs 92,13%. They conclude MALDI TOF MS as a gold standard method. Hone and team undergone study with 212 bacteria and found 92% sensitivity at species level. Panda and his team were carried out study to identify clinical bacteria use of MALDI- TOF –MS in clinical samples and conclude this method is suitable for Clinical lab due to high sensitivity.(Panda et al. 2014).

Even though MALDI –TOF –MS method is rapid, reproducible, pattern specific and more sensitive than PCR method it takes high cost, complex instruments, and more knowledge. PCR method can easily perform with machine like thermos cycler, even without that also it can complete. There are some disadvantage in PCR which leads low sensitivity due to fragmentation of target DNA or damage of DNA during annealing and extension. Ultimately it will not represent original organism. Contamination by other organisms (DNA)

is a major disadvantage in PCR. But MALDI method highly depends on high cost machine and software .Because of single machine out of order whole proses will stop. Also poly microbial sample difficulty to detect in MALDI. Szabados 2011and team studied about scour value with clinical bacteria and conclude vary little information available at species level identification in software. Therefore, clinical lab tends to choose PCR method than MALDI-TOF.

Identification of Bifidobacteria up to species level is less important in clinical diagnostic sector as it is human GI tract and vaginal commensal. But it is impotent in pharmaceutical industry to make probiotic .Bifidobacteria that is used as treatment ford diarrhea, irritable bowel syndrome, Helicobactor pylori infection, and to reduce body cholesterol level.(Panda et al 2014).

Conclusion

Identification of Bifidobateria gnu's level in both methods has given equal and highly accurate result. Identification of species in Bifidobacteria MALDI –TOF-MS is more accurate and a rapid method.

Recommendation

PCR method can improve to identify of Bifid bacteria up to species level with use of species level primers .Apart from that improve DNA purification from direct clinical sample than use cutler colony. MALDI method with formic acid exaction can improve sensitivity with purification of protein. Also accelerate database and improve soft were to detect rare organisms. Cost, complexity should be minimized in both method.

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