

**IDENTIFICATION OF BACTERIAL COMMUNITY IN MILK OF MASTITIC
WATER BUFFALO IN SELECTED COOPERATIVES OF THE NATIONAL
IMPACT ZONE USING PCR AND DGGE METHOD**

NIÑA RICA A. PAGADUAN

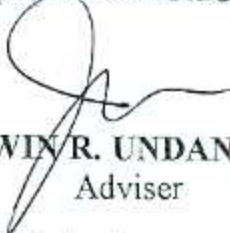
An Undergraduate Thesis Submitted to the Faculty of the Department of Biological
Sciences, College of Arts and Sciences, Central Luzon State University,
Science City of Muñoz, Nueva Ecija, Philippines
in Partial Fulfillment of the Requirements
for the Degree of

**BACHELOR OF SCIENCE IN BIOLOGY
(Major in Microbiology)**

JUNE 2018

ACCEPTANCE SHEET

This undergraduate thesis entitled “IDENTIFICATION OF BACTERIAL COMMUNITY IN MILK OF MASTITIC WATER BUFFALO IN SELECTED COOPERATIVES OF THE NATIONAL IMPACT ZONE USING PCR AND DGGE METHOD” prepared and submitted by NIÑA RICA A. PAGADUAN, in partial fulfillment of the requirements for the degree of BACHELOR OF SCIENCE IN BIOLOGY (MICROBIOLOGY), is hereby accepted.


JERWIN R. UN DAN, Ph.D.
Adviser

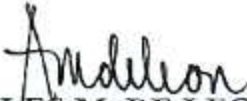
06/13/18

Date Signed


CLARO N. MINGALA, Ph.D.
Co-Adviser

06/13/18

Date Signed


ANGELES M. DE LEON, Ph.D.
Critic

06/13/18

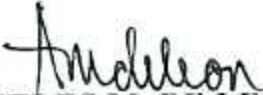
Date Signed


RICH MILTON R. DULAY, M.Sc.
Department Research Coordinator

06/13/18


Date Signed

Accepted as partial fulfillment of the requirements for the degree of BACHELOR OF SCIENCE IN BIOLOGY (MICROBIOLOGY)


ANGELES M. DE LEON, Ph.D.
Chair, Department of Biological Sciences

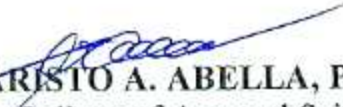
06/13/18

Date Signed


RICH MILTON R. DULAY, M.Sc.
College Research Coordinator

6/13/18

Date Signed


EVARISTO A. ABELLA, Ph.D.
Dean, College of Arts and Sciences

06/13/18

Date Signed

BIOGRAPHICAL SKETCH

The author's name is Niña Rica Anselmo Pagaduan, she is a 20 years old from Ibona, Dingalan, Aurora. She was born on October 29, 1997 in Cabanatuan City and her religion is Pentecost. Her father's name is Arnold P. Pagaduan and her mother's name is Francisca A. Pagaduan. She used to live in Bagong Sikat, Taylan's Residence while studying in Central Luzon State University.

She finished her primary education at Ibona Elementary School from school year 2004-2010, and her secondary at Ibona National High School in Ibona, Dingalan, Aurora from school year 2010-2014. She obtained her tertiary education pursuing Bachelor of Science in Biology major in Microbiology at the Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, in Science City of Munoz and she completed her on the job training in the Ramon Magsaysay- Center for Agricultural Resources and Environmental Studies (RM-CARES) in Science City of Muñoz.

ACKNOWLEDGEMENT

The researcher would like to express her deepest gratitude to those people behind the success in fulfillment of this project. First of all, to the almighty God for giving her wisdom, strength and courage to overcome all the challenges, worship honor and glorify forever.

She would like to thank her adviser Dr. Jerwin R. Undan her adviser, for the kindness, guidance, support and encouragement throughout the study. She would also like to thank Dr. Claro N. Mingala her co-adviser and Dr. Marvin A. Villanueva for providing necessary support and permission to conduct her thesis in Philippine Carabao Center. To Ms. Paula Blanca V. Gaban her supervisor in Biosafety and Environmental Section for her consistent support, kindness, patience and sharing her knowledge to the researcher to successfully finish this paper.

She would also like to give her sincerest thank to her critic, Dr. Angeles M. De Leon and to the Department Research Coordinator Mr. Rich Milton Dulay for their kindness, insightful comments and good suggestions to improve this study.

The researcher also would like to thank to the staffs of the Animal Health and Biosafety and Environmental Section of PCC for assisting her, cheering her and for lending her all the things that she needed to use in her experiments.

To the beloved parents of the researcher, a warm appreciation and gratitude for the financial and moral support that was given to her. And lastly, the researcher wishes to extend her gratitude for those who are not mentioned above that any way helped to fulfil this study.

TABLE OF CONTENTS

	PAGE
LIST OF TABLES	vii
LIST OF FIGURES	viii
LIST OF APPENDICES	ix
LIST OF APPENDIX TABLES	x
LIST OF APPENDIX FIGURES	xi
ABSTRACT	xii
INTRODUCTION	1
Background of the Study	1
Objective of the Study	2
Significance of the Study	2
Scope and Limitation of the Study	3
Time and Place of the Study	3
REVIEW OF RELATED LITERATURE	4
Water Buffalo (<i>Bubalis bubalis</i>)	4
Milk	5
Clinical Mastitis	6
Bacterial Group Associated with Infection	7
Polymerase Chain Reaction	7
16S Rrna	8
DGGE	10
MATERIALS AND METHODS	13
Sampling Sites and Sample Collection	13
DNA Extraction of Milk	14
Polymerase Chain Reaction	15
DGGE	15
Sequencing and Sequence analysis	16
Data Gathered	16
RESULTS AND DISCUSSION	17

Milk Samples Collection	17
16S rRNA Amplification	18
DGGE Analysis	20
Sequencing Results	25
SUMMARY CONCLUSION AND RECOMMENDATION	30
Summary	30
Conclusion	30
Recommendation	31
LITERATURE CITED	33
APPENDICES	39

LIST OF TABLES

TABLE		PAGE
1	List of Selected Cooperatives from NIZ and Number of Samples Collected	17
2	BLAST Identification Results	26

LIST OF FIGURES

FIGURE		PAGE
1	Map of Nueva Ecija	14
2	Amplified PCR product of cooperatives in pulong buli, eastern, CAMPC and some of gene pool	19
3	Amplified PCR product of cooperatives in gene pool (w/Licaong), SIPBU-Porais, minabuyok and riverside	19
4	Amplified PCR product of cooperatives in riverside, SIPBU-Porais, SIPBU- Villa Joson and cinense	19
5a	Denaturing gradient gel electrophoresis of amplified 16S rRNA product of samples from eastern (lanes 1, 2 and 9), SIPBU-Porais (lanes 3 and 4), SIPBU-Villa Joson (lanes 5 and 6), riverside (lanes 7 and 8), pulong buli (lane 10) and gene pool (lanes 11 to 13)	20
5b	Denaturing gradient gel electrophoresis of amplified 16S rRNA product of samples from gene pool-Licaong (lanes 1 and 2), minabuyok (lanes 3, 4 and 5) and CAMPC (lanes 6 and 7).	21

LIST OF APPENDICES

APPENDIX		PAGE
A	List of Milk Samples in Selected Cooperatives of NIZ	40
B	Photo Documentation	44

LIST OF APPENDIX TABLES

APPENDIX TABLE		PAGE
1	Milk samples from Pulong Buli	40
2	Milk samples from Eastern	40
3	Milk samples from CAMPC	40
4	Milk samples from Gene Pool	41
5	Milk samples from SIPBU-Porais	41
6	Milk samples from Minabuyok	41
7	Milk samples from Gene Pool (Licaong)	41
8	Milk samples from Riverside	42
9	Milk samples from SIPBU- Porais	42
10	Milk samples from SIPBU-Villa Joson	42
11	Milk samples from Cinense	43

LIST OF APPENDIX FIGURES

APPENDIX FIGURE		PAGE
1	Twenty ml of milk samples	44
2	DNA Extraction Kit	44
3	Centrifuge	44
4	Microcentrifuge	45
5	Preparation of PCR mixture	45
6	PCR Machine	45
7	Preparation of 1% agarose gel	46
8	Gel Electrophoresis Tank	46
9	DGGE Tank	46
10	Gel Doc	46

ABSTRACT

PAGADUAN, NIÑA RICA A., Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Munoz, Nueva Ecija, Philippines, **JUNE 2018, IDENTIFICATION OF BACTERIAL COMMUNITY IN MILK OF MASTITIC WATER BUFFALO IN SELECTED COOPERATIVES OF THE NATIONAL IMPACT ZONE USING PCR AND DGGE METHOD**

Adviser: JERWIN R. UNDAN, Ph. D.

This study was conducted to determine and identify the presence of bacteria in mastitic milk of water buffalo in selected cooperatives of NIZ using PCR and DGGE method. The milk samples collected from selected cooperatives wherein PCC was conducting the regular milk testing and only those samples which were positive in CMT were used prior to the extraction of DNA. Milk extraction of DNA was done and 16S bacterial primers were used to amplify the target region of 16S rRNA. The amplified PCR products were resolve in DGGE and the bands were excised prior to sequencing in order to identify the bacteria present in the samples.

The identified bacteria in BLAST were all gram-positive bacteria. Among these identified strains of bacteria genus of *Bacillus* was most dominant, genus of *Staphylococcus* and *Kurthia* were second most dominant. These bacteria were commonly found in soil and caused infections. The prevalence of these bacteria could be associated with the handling practices conducted in every cooperative. The identification of the presence of these bacteria can lead to development of drugs used for aiding mastitic in water buffalo and can be used in assessing the proper sanitation and handling practices in any dairy farms. The used of DGGE method was very useful in rapid detection of

bacterial communities in a sample, however it has limitations to be considered as a molecular technique.

LITERATURE CITED

- Badley, A., Leach, K., Breen, J., Green, L. & Green M. (2007). Survey of the incidence and aetiology of mastitis on dairy farms in England and Wales. *Veterinary Record*, 160, 253-258.
- Baloch, H., Rind, R., Kalhoro, D. & Kalhoro, A.B. (2013). Study on Mastitis in Buffaloes Caused by Bacterial Species. *Pakistan Journal of Agricultural Sciences*, 29(2), 83-93.
- Boor, K.J., Brown, D.P., Murphy, S.C. & Bandler, D.K. (1998). Microbial and chemical quality of raw milk in New York State. *Journal of Dairy Science*, 81, 1743-1748.
- Borghese, A. & Mazzi, M. (2005). Buffalo Population and Strategies in the World. *REU Technical Series*, 67, 1-39.
- Bradford, A. (2014). Buffalo Facts: Water Buffalo and Cape Buffalo. Live Science. Retrieved from www.livescience.com
- Chifiriuc, M., Gheorge, I., Czobor, I., Florea, D., Mateescu, L., Caplan, M., Caplan, D. & Lazar, V. (2017). Advances in molecular biology based assays for the rapid detection of food microbial contaminants. *Nanotechnology in the Agri-Food Industry*, 1, 645-669.
- Clarridge III, J. E. (2004). Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, 17, 840-862.
- Coenye, T. & Vandamme, P. (2003). Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiology Letters*, 228(1), 45-9.
- Devriese, L.A. (1990). *Staphylococci* in healthy and disease animals. *Journal Applied Bacteriology*, 69, 71-80.
- D'ambrosio, C., Arena, S., Salzano, A.M., Renzone, G., Ledda, L. & Scaloni, A. (2008). A proteomic characterization of water buffalo milk fractions describing PTM of major species and the identification of minor components involved in nutrient delivery and defense against pathogens. *Proteomics*, 8(17), 3657-3666.
- Ehling-Schulz, M., Messelhauser, U. & Granum, P. (2011). *Bacillus cereus* in Milk and Dairy Production. In Hoofar J (ed), *Rapid Detection, Characterization, Enumeration of Foodborne Pathogens*, 1, 275-289.

- El-Zubeir, I. & Ahmed, M. (2007). The hygienic quality of raw milk produced by some dairy farms in Khartoum-Sudan. *Journal of Microbiology*, 2, 988-991.
- Ercolini, D., Moschetti, G., Blaiotta, G. & Coppola, S. (2001). The potential of a polyphasic PCR-DGGE approach in evaluating microbial diversity of natural whey cultures for water-buffalo mozzarella cheese production: bias of culture dependent and culture-independent analyses. *Systematic and Applied Microbiology*, 24, 610-617.
- Fagiolo, A. & Lai, O. (2007). Mastitis in Buffalo. *Italian Journal of Animal Science*, 6(2), 200-206.
- From, C., Hormazabal, V. & Granum, P. (2007). Food poisoning associated with pumilacidin-producing *Bacillus pumilus* in rice. *International Journal of Food Microbiology*, 115, 319-324.
- Garcia-Pichel, F., Prufert-Bebout, L. & Muyzer, G. (1996). Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium. *Applied and Environmental Microbiology*, 62, 3284-3291.
- Giannino, M., Marzotto, M., Dellaglio, F. & Feligni, M. (2009). Study of microbial diversity in raw milk and fresh curd used for Fortina chees production by culture-independent methods. *International Journal of Food Microbiology*, 130, 188-195.
- Green, S.J., Leigh, M.B. & Neufeld, J.D. (2010). Denaturing Gradient Gel Electrophoresis (DGGE) for Microbial Community Analysis. *Handbook of Hydrocarbon and Lipid Microbiology*, 1, 4137-4158.
- Griffioen, K., Hop, G., Holstege, M., Velthuis, A. & Lam, T. (2016) Dutch Mastitis Diagnostics Consortium Dutch Dairy farmers' need for microbiological mastitis diagnostics. *Journal of Dairy Science*, 99, 5551-5561.
- Halasa, T., Huijps, K., Osteras, O. & Hogeveen, H. (2007) Economic effects of bovine mastitis and mastitis management: A review. *Veterinary Quarterly Journal*, 29(1), 18-31.
- Hamann, J. (2002). Relation between somatic cell count and milk composition. *International Dairy Federation Bulletin*, 372, 56-59.
- Han, X., Lee, F.L., Zhang, L. & Guo, M.R. (2012). Chemical composition of water buffalo milk and its low-fat symbiotic yogurt development. *Functional Foods in Health and Disease*, 2(4), 86-106.
- Han, K., Jung, Y. & Son, S. (2003). Phylogenetic analysis of phenanthrene-degrading *Sphingomonas*. *Journal of Microbiology and Biotechnology*, 13, 942-948.

- Haug, A., Hostmark, A.T. & Harstad, O.M. (2007). Bovine milk in human nutrition – a review. *Lipids in Health and Disease*, 6, 25.
- Jenkins, C., Ling, C., Ciesielczuk, H., Lockwood, J., Hopkins, S., Mchugh, T., Gillespie, S. & Kibbler, C. (2012). Detection and identification of bacteria in clinical samples by 16S rRNA gene sequencing: comparison of two different approaches in clinical practice. *Journal of Medical Microbiology*, 61, 483-488.
- Joo, G., Kim, Y., Lee, I., Song, K. & Rhee, I. (2004). Growth promotion of red pepper plug seedlings and the production of gibberellins by *Bacillus cereus*, *Bacillus macrolides* and *Bacillus pumilus*. *Biotechnology Letters*, 26, 487-491.
- Keddie, R. (1981). The prokaryotes. *A handbook on habitats, isolation and Identification of bacteria (1 ed.)* Springer-Verlag Berlin Heidelberg, 1, 1888-1893.
- Kerremans, J., Verboom, P., Stijen, T., Hakkaart-van Roijen, L., Goessens, W., Verburg, H. & Vos, M. (2008). Rapid Identification and antimicrobial susceptibility testing reduce antibiotic use and accelerate pathogen-directed antibiotic use. *Journal of Antimicrobial Chemotherapy*, 61, 428-435.
- Kimouli, M., Vrioni, G. & Papadopoulou (2012). Two cases of severe sepsis caused by *Bacillus pumilus* in neonatal infants. *Journal of Medical Microbiology*, 61(4), 596-599.
- Kolbert, C.P & Persing, D. H. (1999). Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Current Opinion in Microbiology Journal*, 2(3), 299-305.
- Kuang, Y., Tani, K., Synnott, A., Ohshima, K., Higuchi, H. Nagahata, H. & Yasunori T. (2009). Characterization of Bacteria Population of raw milk from bovine mastitis by culture-independent PCR-DGGE method. *Biochemical Engineering Journal*, 45, 76-81.
- Levison, L., Miller-Cushon, E., Tucker, A., Bergeron, R., Leslie, K. & Barkema H. (2016). Incidence rate of pathogen-specific clinical mastitis on conventional and organic Canadian dairy farms. *Journal of Dairy Science*, 99(2), 1341-50.
- Marsh, T. L. (1999). Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Current Opinion in Biotechnology*, 2, 323-327.
- McAuliffe, L., Ellis, R.J., Lawes, J.R., Ayling, R.D. & Nicholas, R.A. (2005). 16S rDNA PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. *Journal of Medical Microbiology*, 54(8), 731-739.

- Mincheol, K. & Chun, J.S. (2014). 16S rRNA Gene-Based Identification of Bacteria and Archaea using the EzTaxon Server. *Method in Microbiology*, 41, 61-74.
- Mullis, K.B. & Faloona, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase catalysed chain reaction. *Methods Enzymology Journal*, 155, 335-350.
- Muyzer, G. (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology*, 2, 317-322.
- Muyzer, G., De Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, *Applied and Environmental Microbiology*, 59, 695-700.
- Muyzer, G. & Smalla, K. (1998). Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoe*, 73, 127-141.
- Nickerson, S.C. (2009). Control of heifer mastitis: antimicrobial treatment-an overview. *Veterinary Microbiology*, 134(1-2), 128-35.
- Nubel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I. Ludwig W. & Backhaus, H. (1996). Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology*, 178, 5636-5643.
- Oliver, S.P., Jayarao B.M. & Almeida R.A. (2005). Food borne pathogens in milk and the dairy farm environment. Food safety and public health implication. *Foodborne Pathogen and Disease*, 2, 115-129.
- Ovreas, L. & Torsvik, V. (1998). Microbial Diversity and Community Structure in Two Different Agricultural Soil Communities. *Microbial Ecology*, 36, 303-315.
- Pan, Z., Li, L., Shen, Z. & Li, M. (2018). Characterization of the microbiota in air and vacuum-packed crisp grass carp (*Ctenopharyngodon idella* C. et V.) Fillets by 16S rRNA PCR-Denaturing Gradient Gel Electrophoresis and High-throughput sequence. *Journal of Food Protection*, 15, 1022-1029.
- Parkinson, T., Merrall, M. & Fenwick, S.G (1999). A case of bovine mastitis by *Bacillus cereus*. *New Zealand Veterinary Journal*, 47(4), 151-152.
- Patel, J. B. (2001). 16S rRNA gene sequencing for bacterial pathogen identification in the clinical laboratory. *Journal of Molecular Diagnostics*, 6, 313-321.

- Piccinini, R., Miarelli, M., Ferri, B., Tripaldi, C., Belotti, M., Dapra, V., Orlandini S. & Zeconi, A. (2006). Relationship between cellular and whey components in buffalo milk. *Journal of Dairy Research*, 73, 129-133.
- Pitkala, A., Gidonis, V. Wallin, H. & Honkanen-Buzalski, T. (2005). Interlaboratory proficiency testing as tool for improving performance in laboratories diagnosing bovine mastitis. *Journal of Dairy Science*, 88, 553-559.
- Potekhina, N. (2011). Phosphate containing cell wall polymers of *Bacilli*. *Journal of Biochemistry*, 7(6), 745-754.
- Priest, F.G. (1993). Systematics and Ecology of *Bacillus*. *Bacillus subtilis* and other Gram-Positive Bacteria: Biochemistry, Physiology and Molecular Genetics. *American Society for Microbiology Press*, 3, 16.
- Reikerink, R., Barkema, H., Kelton, D. & Scholl, D. (2008). Incidence rate of clinical mastitis on Canadian dairy farms. *Journal of Dairy Science*, 91(4), 1366-77.
- Rochelle, P., Cragg, B., Fry, J., Parkes, R. & Weightman, A. (1994). Effect of sample handling on estimation of bacterial diversity in marine sediments by 16S rRNA gene sequence analysis. *FEMS Microbial Ecology*, 15, 215-226.
- Salman, A. & Hamad, I. (2011). Enumeration and identification of Coliform bacteria from raw milk in Khartoum State, Sudan. *Journal of Cell and Animal Biology*, 5(7), 121-128.
- Shakoor, A., Athar, M., Muhammad, G., Rahman, S. U., Butt, A. A., Hussain, I. & Ahmad, R. (2006). Experimental trials of live attenuated and inactivated *Staphylococcus aureus* vaccines in Rabbits. *Pakistan Veterinary Journal*, 26 (2), 51-54.
- Spencer, R.C. (2003). *Bacillus anthracis*. *Journal of Clinical Pathology*. 56(3), 182-187.
- Stakerbrandt, E., Keddie, R. & Jones, D. (2006). The Genus *Kurthia*. *Journal of Royal Society Interface*, 4, 519-529.
- Stepanovic, S., Vukovic, D., Trajkovic, V., Samardzic, T., Cupic, M. & Svabic-Vlahovic, M. (2001). Possible virulence factors of *Staphylococcus sciuri*. *FEMS Microbiology Letters*, 199, 47-53.
- Strathdee, F. & Free, A. (2013). Denaturing Gradient Gel Electrophoresis (DGGE). *Methods in Molecular Biology Journal*, 1054, 145-157.
- Suzuki, M. & Giovannoni, S. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Applied Environmental Microbiology*, 62, 625-630.

- Tzeneva, V., Heilig, H., Van Vliet, W., Akkermans, A., De Vos, W. & Smidt, H. (2008). 16S rRNA targeted DGGE fingerprinting of microbial communities. *Methods of Molecular Biology*, 410, 335-349.
- Tzukuda, M., Kithara, K. & Miyazaki, K. (2017). Comparative RNA function analysis reveals high functional similarity between distantly related bacterial 16S rRNAs. *International Journal of Scientific Reports*, 7(1), 9993.
- Vakkamaki, J., Taponen, S., Heikkilä, A. & Pyörälä, S. (2017). Bacterial etiology and treatment of mastitis in Finnish dairy herds. *Acta Veterinaria Scandinavica*, 59, 33.
- Valskova, V. & Baldrian, P. (2009). Denaturing gradient gel electrophoresis as a fingerprinting method for the analysis of soil microbial communities. *Plant Soil Environment Journal*, 55(10), 413-423.
- Vanderhaeghen, W., Piepers, S., Leroy, F., Van Coillie, E., Haesebrouck, F. & De Vliegher, S. (2014). Effect, persistence and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. *Journal Dairy Science*, 7, 5275-5293.
- Vos, P., Garrity, G., Jones, D., Krieg, N.R., Ludwig, W., Rainey, F.A. & Whitman, W.B. (2009). The Firmicutes. *Bergey's manual of systematic bacteriology*, 3, 13.
- Werner, P., Cowie, I. & Cusack, J. (2014). Juvenile tree growth and demography in response to feral water buffalo in savannas of Northern Australia: an experimental field study in Kakadu National Park. *Australian Journal of Botany*, 54(3), 283-296.
- Wheeler, A. & Stahl, D. (1996). Extraction of microbial DNA from aquatic sediments. *Molecular Microbial Ecology Manual*, 1, 1-29.
- White, D., Walmsley M., Liew, A., Claycomb, R. & Mein, G. (2005). Chemical and rheological aspects of gel formation in the California Mastitis Test. *Journal of Dairy Research*, 72, 115-121.
- Yamane, I. (2006). Epidemiological survey and economical evaluation on bovine mastitis in tie-stall dairy farms, *Journal of Japan Veterinary Medicine Association*, 59, 674-678.