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Editorial Statement

Pertanika is the official journal of Universiti Putra Malaysia. The abbreviation for Pertanika Journal of Tropical Agricultural Science is *Pertanika J. Trop. Agric. Sci.*

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EDITORIAL OFFICE

JOURNAL DIVISION
Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 Serdang, Selangor Malaysia.
Gen Enq.: +603 8947 1622 | 1619 | 1616
E-mail: executive_editor.pertanika@upm.my
URL: www.journals-jd.upm.edu.my

PUBLISHER

Kamariah Mohd Saidin
UPM Press
Universiti Putra Malaysia
43400 UPM, Serdang, Selangor, Malaysia.
Tel: +603 8946 8855, 8946 8854
Fax: +603 8941 6172
E-mail: penerbit@putra.upm.edu.my
URL: http://penerbit.upm.edu.my

ABSTRACTING/INDEXING

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Foreword

Welcome to the **First Issue 2014** of the Journal of Tropical Agricultural Science (JTAS)!

JTAS is an open-access journal for the Tropical Agricultural Science published by Universiti Putra Malaysia Press. It is independently owned and managed by the university and run on a non-profit basis for the benefit of the world-wide science community.

This issue contains **12 articles,** out of which **one** is a review article and **11** are regular research papers. The authors of these articles are from **Malaysia, Nigeria, Australia** and **Iraq**.

The review paper discusses chromosomal anomalies and infertility in farm animals (*Yimer, N. and Rosnina, Y.*). The authors mention that chromosome abnormalities in farm animals can cause significant adverse effects on fertility through failure of production of viable gametes or early embryonic death, both of which lead to great economic loss. The paper discusses several strategies to prevent and control the propagation of chromosomal aberrations in farm animals, especially in the tropics.

The 11 research papers cover a wide range of topics. In the first research paper, researchers from Universiti Putra Malaysia investigate the potential of *Polymesoda erosa* as a biomonitor and food safety concern (*Yap, C. K., Edward, F. B. and Tan, S. G.*). A research group from Nigeria discusses in detail the correlation and path analyses of seed yield in okra (*Abelmoschus esculentus* (L.) *Moench*) grown under different cropping seasons (*Adekoya, M. A., Ariyo, O. J., Kehinde, O. B. and Adegbite, A. E.*). In the next research paper, the light and scanning electron microscopy of the small intestine of young Malaysian village chicken and commercial broiler is reported (*Khalid K. Kadhim, Md Zuki Abu Bakar, Mohamed Mustapha Noordin, Mohd Amin Babjee and Mohd Zamri Saad*).

Another group of researchers from Nigeria reports on morphological and leaf epidermal features of some Capsicum species (Solanaceae) from Nigeria (*Aworinde, D. O., Ogundele, A.* and *Ogundairo, B. O.*), while a group of researchers from Universiti Kebangsaan Malaysia reports on the ongoing human-macaque conflict on the main campus of Universiti Kebangsaan Malaysia (*Md-Zain, B. M., Ruslin, F. and Idris, W. M. R.*). From Universiti Sains Malaysia, a group of researchers completes the amphibian checklist of Bukit Larut, Perak, Malaysia (*Shahriza Shahrudin* and *Ibrahim Jaafar*).

Members of the Federal University of Agriculture, also from Nigeria, describe carcass and meat characteristics of traditionally managed Nigerian *yankasa* and West African dwarf breeds of sheep (*Fasae O. A., Oduguwa B. O., Adejumo L. A. Makinde T. E. and*

Sanwo K. A.), while collaboration among researchers from three countries, Australia, Malaysia and Iraq, sheds light on the impact of Xylanase supplement on the bacterial community of a chicken's intestinal tract (*Samsudin, A. A. and Al-Hassani, D. H.*).

Subsequent papers describe the antioxidant properties of two varieties of bitter gourd (*Momordica charantia*) and the effect of blanching and boiling on them (*Choo, W. S., Yap, J. Y.* and *Chan, S. Y.*); the successful induction of skin ulcers in moon light gourami (*Trichogaster Microlepis*) with *Aphanomyces Invadans* Zoospores (*Afzali, S. F., Hassan, M. D. and Mutalib A. R.*); and the isolation and characterisation of *Lactobacillus* strains as potential probiotics for chickens (*Shokryazdan, P., Kalavathy, R., Sieo, C. C., Alitheen, N. B., Liang, J. B., Jahromi, M. F. and Ho, Y. W.*).

I anticipate that you will find the evidence presented in this issue to be intriguing, thought-provoking and useful in reaching new milestones in your own research. Please recommend the journal to your colleagues and students to make this endeavour meaningful.

I would also like to express my gratitude to all the contributors, namely, the authors, reviewers and editors who have made this issue possible. Last but not least, the editorial assistance of the journal division staff is fully appreciated.

JTAS is currently accepting manuscripts for upcoming issues based on original qualitative or quantitative research that opens new areas of inquiry and investigation.

Chief Executive Editor
Nayan Deep S. KANWAL, FRSA, ABIM, AMIS, Ph.D.
nayan@upm.my

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Review Paper

Chromosomal Anomalies and Infertility in Farm Animals: A Review

Yimer, N.* and Rosnina, Y.

Theriogenology and Cytogenetics Unit, Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Veterinary cytogenetics is an area of genetics that deals with normal or abnormal chromosomes of animals. Chromosome abnormalities in cattle can cause significant adverse effects on fertility through failure of production of viable gametes or early embryonic death that consequently leads to great economic loss. Chromosomal aberrations can occur as numerical errors or structural rearrangements usually without causing phenotypic abnormalities on carrier animals. According to current knowledge on chromosomal abnormalities, Robertsonian translocation (ROB) that involves chromosome 1 and 29 represents the most common form of aberration found in cattle of various breeds. Other less commonly encountered abnormalities in cattle include reciprocal translocation, chimerism (including freemartins), mosaic and rarely sex chromosome aberrations. A similar trend in incidence of abnormalities has been observed in sheep and goats although systematic studies are limited. Centric fusion that involves different chromosomes but not specific to chromosome (1;29), is the most common abnormality, followed by chimerism, sex chromosome abnormalities, and rarely deletions and inversions. In swine, reciprocal translocations are the most common abnormalities with significant economic loss due to reduction in litter size by up to 50%. This is followed by chimerism for sex chromosomes. Unlike cattle, incidence of ROB (1,29) in pigs is very rare. In domestic buffaloes, sex chromosome abnormalities are the most common found associated with infertility due to extensive damage to sex adducts. Freemartinism is the most prevalent form of all sex chromosome abnormalities detected in buffalo. However, translocations are rare incidences in buffalo. Sound breeding programmes and successful cattle production depend on minimizing

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E-mail addresses:
nurdeg2006@gmail.com (Yimer, N.),
yrosnina@yahoo.com (Rosnina, Y.)
* Corresponding author

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and preventing all kinds of possible causes of reproductive failure in both females and males. Unlike many other causes of reproductive failure, little attention appears to be given towards cytogenetic anomalies examination at the time of breed selection for genetic improvement, as well as during investigation of causes of reproductive failure in farm animals. This is especially true in the tropical regions where cytogenetic studies are limited and their significance is poorly understood. This review provides an overview and update on chromosomal anomalies, their effect on fertility of farm animals and screening methods, with subsequent aim of drawing the attention of concerned bodies to make an effort towards understanding the magnitude and significance of the problem in the tropics by applying feasible and available biotechnological tools pertinent to cytogenetics. This consequently would help to design possible strategies to prevent and control the propagation of chromosomal aberrations in farm animals in the tropics.

Keywords: Chromosomal anomalies, cytogenetics, farm animals, reproductive failure, screening techniques

INTRODUCTION

Maintaining high fertility rate and economic return is the main goal of animal farming enterprises. Fertility is a multifactorial phenomenon which is influenced by several factors that often overlap including nutrition, disease, hormonal disturbance, reproductive functions, sperm quality, physical soundness, environment, management and genetics. Cattle breeders are well aware of the effect of most of these factors and take measures to minimise associated problems at the time of animal selection. However, it is very rare to see breeders much worried about genetic abnormalities related to chromosomes especially in areas where cytogenetic studies are limited such as in the tropics including South East Asia and Africa. The magnitude and significance of occurrence of chromosome anomalies are also little understood. Although reduced fertility occurs mostly due to a combination of several factors, in some instances, it can be traced to a single genetic factor. For example, with regard to chromosome anomalies, bulls with Y-autosome reciprocal translocations (Iannuzzi *et al*., 2001) show an apparent normal phenotype but found with oligozoospermia or azoospermia. Thus, it is always important to consider cytogenetic anomalies as possible causes of reproductive failure during investigation and keep updated on their occurrence, distribution, significance and related techniques for investigation.

CYTOGENETIC ANOMALIES AND THEIR EFFECT ON FERTILITY OF FARM ANIMALS

Cattle

Domestic cattle of *Bos taurus*, *Bos indicus* and their crosses normally possess a diploid number of 60 chromosomes, which comprises 29 pairs of autosomes and a pair of sex chromosomes (XX in females and XY in males). Structurally, all the 29

pairs of autosomes and the X chromosome are acrocentric and sub-metacentric, respectively, in both *Bos taurus* and *Bos indicus* breeds. The only difference is in the Y chromosome, which is sub-metacentric in *Bos taurus* but acrocentric in *Bos indicus* breeds (Lightner, 2008).

Chromosomal abnormalities can occur as a result of alterations in number such as polyploidy (the condition of having the entire set of chromosomes beyond the basic set with an exact multiple of the haploid number) and aneuploidy (the condition of having or missing one or more chromosomes from the basic set), or as a result of structural changes which consist of translocations, deletion, duplication and inversion. Such chromosomal abnormalities which involve the chromosome number and structure including their frequency and phenotypic effects are dealt under clinical cytogenetics (Popescu, 1996). The initiation of conducting clinical cytogenetic investigations is usually associated with observation of fertility problems, congenital defects or as a part of an eradication programme for chromosome aberrations (Iannuzzi *et al.*, 1993; Lioi *et al*., 1995; Popescu, 1996; Molteni *et al*., 1998; 2007; Ducos *et al*., 2000). Variable degrees of infertility in cattle of different breeds, which are carriers of numerical or structural chromosomal abnormalities, have been reported in previous studies (Muñoz *et al*., 1994; Rubes *et al*., 1996; Schmutz *et al*., 1997; Molteni *et al*., 1998; Tanaka *et al*., 2000). Anomalies such as translocations (Gustavsson, 1979; Popescu, 1996; Ducos *et al*., 2000, 2007, 2008), tandem fusion and chimerism for sex cells (Pinheiro *et al*., 1995), sex chromosome aneuploidy (Gustavsson, 1977; Citek *et al*., 2009) are among the important abnormalities in cattle reported to be associated with variable degrees of infertility. Other abnormalities such as deletions and insertions are not commonly found in animals as they are lethal at early stage of an embryonic life.

As it is well recorded, the most frequently reported and widely distributed chromosomal aberration in cattle of various breeds is centric fusion, also known as Robertsonian translocation (ROB). Robertsonian translocation involves the fusion of two acrocentric chromosomes at their centromeres thereby forming a single large sized meta/sub-metacentric chromosome and reducing the total chromosome number by one in heterozygous carriers or by two in homozygous cases. The most prevalent form of ROB is the fusion between the 1st and the 29th chromosomes, ROB (1;29) (Gustavsson, 1979; Schifferli *et al*., 2003; Ducos *et al.*, 2008; De Lorenzi *et al.*, 2012). It was first identified in Swedish Red and White cattle (Gustavsson & Rockborn, 1964). Since then, several other reports have described this translocation in scores of different cattle breeds from all six continents with a considerable variation in incidence among breeds (Popescu & Pech, 1991; Lightner, 2008; Ducos *et al.*, 2008). Both male and female cattle with ROB (1;29) are carriers and appear phenotypically normal (Iannuzzi *et al*., 1993; Rubes *et al*., 1996). However, these animals have variable degrees of infertility because of production

of unbalanced or aneuploid gametes that result in the formation of zygotes with unbalanced chromosomal constituents that subsequently results in early embryonic death (Blazak & Eldridge, 1977; Wilson, 1990; Schmutz *et al*., 1991; Tateno & Miyake, 1994; Popescu, 1996; Molteni *et al*., 2005). In cattle carrying ROB (1;29), the observed value of unbalanced gametes is 2.76% in sperms and 4.06% in oocytes (Bonnet-Garnier *et al*., 2008).

Since Gustavsson (1979) has demonstrated the effect of ROB (1;29) in reducing fertility in heterozygous carrier cattle, several other researchers have also confirmed its adverse effect on fertility based on key reproductive performance indicators at different times (Schmutz *et al*., 1991; Popescu, 1996; Molteni *et al*., 2005; Ducos *et al*., 2008). For instance, Schumtz (1990) stated that bulls carrying ROB (1;29) cause abnormal embryos in about 10% of conceptions, while cows carrying this ROB cause 20% of embryos to be abnormal. A diminished non-return rate of 5 and 7% at 28 and 56 days post insemination, respectively (suggesting embryonic mortality), was reported from females sired by bulls heterozygous for ROB (1;29) (Dyrendahl & Gustavsson, 1979). Similarly daughters of carrier heterozygous bulls have been reported to have reduced fertility and greater culling rate than daughters of normal bulls (Schifferli *et al*., 2003). A 5 – 10% reduction in fertility by increasing the embryonic death has been reported by Popescu (1996). Another study, which compared heterozygous carrier cows for ROB (1;29) with cows having normal karyotype, showed that carrier cows required higher insemination index and resulted in lower fertility characterised by lower percentage of pregnancy following first insemination (Kovacs *et al*., 1992). Moreover, carrier cows were also reported to show delayed age at first breeding and calving, prolonged calving interval, decreased calving rate and prolonged number of days open compared to the normal cows. Although not as frequent as ROB (1;29), several other ROBs were also identified, in which all 29 pairs of chromosomes had been involved in at least one ROB described in the literature (Lightner, 2008). The reduction in fertility in cows was observed not only for ROB (1;29) but also for other forms of ROB translocation such as ROB (26;19) (Ducos *et al.*, 2008). Based on experimental studies, heterozygous cows for ROB (26;19) sired by carrier bull compared with normal cows sired by the same carrier bull showed an increase in percentage of negative service, irregular return to heat as well as increase in average calving interval (Ducos *et al.*, 2008). Heterozygosity for a ROB theoretically leads to formation of normal or balanced gametes and through meiotic nondisjunction to the formation of aneuploid gametes. Participation of such aneuploid gametes in fertilization results in monosomic zygotes (a form of aneuploidy which occurs when one chromosome is absent from the normal diploid number) and trisomic zygotes (a form of aneuploidy which occurs when there are three copies of a chromosome instead of the normal diploid number) which die

during gestation (Blazak & Eldridge, 1977). The magnitude of reproductive disturbances depends on the frequency with which non-disjunction occurs at meiosis, viability of aneuploid gametes and zygotes.

Even though it has not been described as often as ROB, there are some reports on reciprocal translocation (RCT) in cattle which are phenotypically normal but with poor fertility (Ducos *et al*., 2000; Iannuzzi *et al*., 2000) and even with infertility/ azoospermia problems (Molteni *et al*., 2007). Although ROB (1;29) represents the most prevalent abnormality in cattle, there is a debate about the true incidence of RCT. De Lorenzi *et al.* (2012), who investigated real estimate of reciprocal translocations based on 15 years of data on cytogenetic results using mathematical and simulation approach, concluded that only 16% of RCT could be detected using simple Giemsa technique. It was shown that RCT could be present in no less than 0.14% of cattle subjects, a frequency five times higher than the de novo ROBs (that involves chromosomes other than chromosomes 1 and 29) (De Lorenzi *et al.*, 2012). According to Ducos *et al.* (2008), based on a screening programme from Italy, they reported an incidence of 7.1% for ROB and 0.03% of RCT. Out of the 7.1% of ROB incidences, majority were the endemic form (1;29), while the remaining 0.03% of the incidences were due to de novo ROB translocations, a frequency very similar to RCT. This implies the need to employ more efficient cytogenetic techniques such as chromosome banding and molecular techniques in order to avoid failure of detection and subsequent underestimation of the incidence of RCT in cattle. Other less commonly encountered anomalies compared to ROB in cattle include chimerism for sex chromosomes including freemartins and rarely sex chromosome aneuploidy (De Lorenzi *et al.*, 2012). Freemartinism is considered as the common form of sex chromosome anomalies in cattle. In 86.4% of females born co-twin together with males globally presented an XX/XY blood chimerism, while the remaining 13.6% had normal XX karyotype (De Lorenzi *et al.*, 2012).

Most of these chromosomal abnormalities reported so far are mainly from European breeds of cattle. Currently, only the incidence of ROB (1;29) in many breeds of cattle populations in Europe is known. It has been reported as high as 65% incidence in Barrosas breed of Portugal (Figueiredo & Iannuzzi, 1993). Following reports of the incidence of ROB (1;29) and its deleterious effect on fertility in 1979, a control programme was established in many countries of Europe whereby animals intended for breeding are cytogenetically evaluated and selected against chromosomal abnormalities (Popescu, 1996). Investigation and monitoring activities were increased in many countries of the world in order to bring ROB (1;29) under control. Eradication programme of ROB (1;29) carrier bulls in Sweden was introduced in 1969 to eliminate the translocation and has subsequently resulted in increased fertility of the whole population (Gustavsson, 1979). In Australia and Great Britain, cytogenetic evaluation

is a requirement for all cattle imported. In Europe, generally speaking, it has been a requirement for imported breeding animals or semen is free of chromosomal abnormalities.

In contrast, despite the huge cattle populations that exist in the tropics including Asia and Africa, and the poor reproductive performance prevailing, studies on chromosomal abnormalities including ROBs are very limited. There is one published report on incidence of a case of ROB (2,28) in Vietnamise cattle (Tanaka *et al*., 2000). Reports of cytogenetic anomalies including ROBs from Africa with large cattle population are also very few (Pathiraja *et al*., 1985; Nel *et al*., 1985) with no later research investigations available. These studies demonstrated incidence of ROB (1;29) in one of 10 Rahaja bulls of Nigeria and 10.2 % of Nguni indigenous cattle breeds of South Africa, respectively. Although such reports were alarming type for the possible high occurrence in the region, further investigations appeared to be discontinued. As a result, the frequency and significance of chromosomal anomalies remain largely unknown. It is expected that the incidence of chromosomal anomalies might have been also worsened by genetic inheritance and introduction of foreign breeds of cattle/germ plasm into these regions in the absence of a control system for cytogenetic anomalies.

Sheep and Goats

Studies on chromosomal anomalies and screening programmes in small ruminants were not as systematic as they were for cattle. Considering the close similarity in the structure of chromosomes of cattle with goats and sheep, the pattern of incidence in the type of chromosome anomalies might be similar. However, this needs a wider investigation for validation.

Centric fusion is the predominant abnormality in sheep and goats though there is some degrees of variation in terms of breed and number of animals evaluated (Long, 1990; Goncalves *et al*., 1992; Dai *et al*., 1994; Switonski & Stranziger, 1998), with most of the studies limited to less than 100 animals. Among the commonly detected forms of centric fusion including ROBs involve chromosome numbers 5,26; 8,11; 7, 25 in sheep (Dai *et al*., 1994) and 5,15; 6;17, 2,12; 6,15; 10,12; 3,17 in goats, mostly of Saanen breeds and reported mainly from Europe, Israel and Brazil (Goncalves *et al.*, 1992; Switonski & Stranziger, 1998). Unlike cattle, incidence of ROB translocation that involve chromosomes 1;29 in sheep and goat is rare; one report in sheep that belong to the Portuguese Churra Terra Quente breed (Chaves *et al*., 2003). In a cytogenetic study that involved 205 goats related to a carrier male goat to a ROB (5;15), the prevalence of heterozygous carriers found was 29.27%, while 4.88% of them were homozygous carriers for the translocation (Goncalves *et al.,* 1992).

Data on the effects of individual chromosome abnormalities on fertility of sheep and goats are rare. It was reported in sheep that despite several ROB translocations described, reduction in fertility was not

observed (Long, 1997). Nevertheless, presence of association between numerical chromosome abnormalities and reduction in fertility in goats has been shown by Bhatia and Shanker (1996). In this study, goats that showed late maturity, anestrus, repeat breeding problem and not conceiving despite regular estrus were investigated for their chromosomal status and compared with their normally performing counter partners. The results showed that 51% of those animals with reproductive problems exhibited various forms of numerical chromosomal abnormalities such as mosaic for polyploidy (17 diploid/tetraploid mosaic; 2 diploid/triploid/tetraploid mosaic, 3 monosomy/diploid mosaic and 1 chimeric) but none of the control animals exhibited such abnormalities. A higher frequency of leucocyte chimerism for sex chromosomes (XX/XY) was also detected in sheep breeds from Poland (De Lorenzi *et al*., 2012). However, the frequency of freemartinism (XX/XY chimerism) was known to be low in sheep compared to the incidence in cattle and buffalo, despite the common co-twin birth in sheep (Brace *et al*., 2008; Di Meo *et al*., 2010). Other less commonly reported chromosomal abnormalities include sex chromosome abnormalities, deletions and inversions (De Lorenzi *et al*., 2012). As cytogenetic studies in sheep and goat are yet limited, making generalizations as to the magnitude of the various types of abnormalities, their significance and influence on fertility is difficult. Therefore, more systematic cytogenetic investigations involving larger populations should be encouraged for a better conclusion.

Buffaloes

The domestic buffalo (*Bubalus bubalis*) are also among the important farm animals that play major roles in the animal production sector especially in the Asian continent. In India, for example, dairy buffaloes contribute 60% of the total milk produced by the dairy industry (Chauhan *et al.,* 2009); hence, they are of great economic importance. The domestic buffalo have been classified into two subspecies. The first type is the river buffalo raised in most areas from India to Egypt and some South and East Europe. The second type is the swamp buffalo of the South East Asia (Bongso & Hilmi, 1982; Iannuzzi, 2007; Supanuam *et al.,* 2009). The chromosome complement of the two types of buffalo and their crossbreed was described using Giemsa and centromeric banding techniques by Bongso and Hilmi (1982). The diploid chromosome number of the River buffalo (Murrah) is 50, while that of the Swamp type (Malaysian Kerbau) is 48. The F_1 hybrid between the two buffalo types has 49 chromosomes. It was also described that the largest two metacentric chromosomes of the Swamp buffalo is the result of a tandem fusion between the telomere of the short arms of chromosome 4 and the centromere of chromosome 9 of the River buffalo (Bongso & Hilmi, 1982; Hishunuma *et al.,* 1992).

When standard nomenclature for chromosomes of most domestic species of animals was established in the 1980s and later revised in the 1990s, the swamp and the river buffaloes were not considered

among the species. Hence, cytogenetic studies during that time were focused on understanding the normal chromosome structure and description of the region of the tandem fusion in chromosome number one of the swamp type towards the establishment of standard karyotype (Hishunuma *et al*., 1992) but with little attention given to clinical cytogenetics. Clinical cytogenetics for investigation of chromosomal anomalies in buffaloes was developed much later compared to other domestic animals (Iannuzzi *et al*., 2005; Iannuzzi, 2007; Di Meo *et al*., 2008; Chauhan *et al.*, 2009). Unlike cattle, systematic cytogenetic screening programmes for chromosome anomalies in both river and swamp buffaloes are rare and most of the reports are individual cases or from a group of animals selected for reproductive problems. The most common chromosome abnormality reported in buffalo (mostly from river type) is sex chromosome abnormality which includes X-trisomy (Parakash *et al.,* 1994; Iannuzzi *et al*., 2004), X-monosomy (Iannuzzi, 2000), Sex-reversal syndrome (Iannuzzi *et al.*, 2001; 2004), XXY-syndrome (Patel *et al*., 2006) and XX/XY mosaicism (freemartinism) (Iannuzzi *et al.*, 2005). Nevertheless, unlike cattle, autosomal chromosome abnormalities such as translocations have rarely been reported in river buffalo.

Almost all the sex chromosome abnormalities reported in river buffalo were associated with sterility due to damage to the internal sex adducts of the females (Iannuzzi, 2007) and inability to produce sperm (azoospermia) in males (Chauhan *et al*., 2009). Freemartinism (XX/XY chimerism) is the most common sex chromosome abnormality in river buffalo as in cattle. For example, in a study that involved 134 river buffaloes with reproductive problems after maturity (13 males, 2 male co-twins and 119 females which had failed to become pregnant in the presence of bulls), 25 animals (20.7%) were found to carry sex chromosome abnormalities. Of which, majority (20, 18 females and 2 males) of them were XX/XY free-martins and all the female carriers were sterile (Di Meo *et al.*, 2008). In another study by Iannuzzi *et al*. (2005) from Italy, it was also shown that freemartinism as the major sex chromosome error found in river buffaloes with reproductive problems. Of the 42 animals studied, 10 freemartins (8 females and 2 males) were found. Six of the eight females showed normal body conformation, vagina and clitoris, while two showed some male traits (tight pelvis). The two males were apparently normal with only a reduced size of one testicle in one animal. Based on clinical observations performed in the internal reproductive organs of the female carriers, serious damages varying from complete lack of internal sex adducts (closed vagina) to hypoplasia of Müllerian ducts and absence (or atrophy) of ovaries were revealed (Iannuzzi *et al.*, 2005). According to histological examination of Murrah and Swamp buffalo hybrids, it has been also revealed that a large proportion of degenerating spermatocytes and abnormal spermatids in the process of spermatogenesis

leading to unbalanced gametes. These unbalanced meiotic products may probably lead to selection against such spermatozoa or early embryos after fertilization causing subnormal fertility (Bongso *et al.*, 1983).

Almost all examined XX/XY freemartin cases in buffalos were from single births, which means that the male co-twin dies during the early embryonic life and a single female birth arises (Iannuzzi, 2007). This makes it difficult to suspect the single born female of being a freemartin and will be allowed to stay in the farm until sexual maturity and show reproductive problems causing great economic loss. This indicates the importance of extending studies to all females (born single or co-twinned) with reproductive problems. Moreover, to minimize the economic loss due to raising a sterile female by waiting until it reaches the age of maturity and manifests reproductive problems, early clinical examination and detection aided by male traits expressed by many of carrier females and subsequent cytogenetic evaluation should be encouraged for prompt elimination from the farm.

Pigs

The pig is the most extensively evaluated animal for which accurate estimate of the prevalence of structural chromosomal anomalies is available (De Lorenzi *et al.*, 2012). An estimated 0.47% prevalence of balanced chromosomal rearrangements was recorded from a large number of young boars karyotyped (Ducos *et al*., 2007). In pigs, where chromosome banding techniques used to be applied as screening method, RCT is the most frequent form of chromosomal anomaly encountered. There are 33 different RCTs known worldwide (mostly reported from Europe), which are responsible for significant economic loss due to a reduction in litter size by 30 - 50% (Ruth *et al*., 1993; Rodriguez *et al*., 2010). Early embryonic mortality accounted for the reduction in litter size (Popescu *et al*., 1984). Chimerism for sex chromosomes is the second most commonly reported abnormality in pigs associated with hypoprolificity and intersexuality, while ROBs were rarely detected (Ducos *et al.,* 2004; Ducos *et al.*, 2008) unlike cattle. In human newborns, where more advanced screening methods used to be involved, the frequency of ROB and RTC are reported to be very similar (Van Assche *et al.*, 1996) and their frequencies were higher in couples experiencing repeated pregnancy losses (De Braekeleer & Dao, 1991).

Although the impact of chromosomal translocations on fertility is widely acknowledged, there exists some degree of differences in terms of species and type of translocation. In pigs, according to several studies, the proportion of unbalanced sperm ranged between 47.83 and 24.33% depending on RCT type (Pinton *et al*., 2004). The proportion of chromosomal unbalanced sperm produced by translocation carriers in humans ranged from 19% to more than 80%, which appeared to be again dependent on the type of translocation (Benet *et al*., 2005). According to Ferguson *et al*. (2008), it has been shown that RCT can induce failure in synaptonemal complex and

arrest of meiosis process. These imply that the impact on fertility posed by RCP is much higher than ROB, possibly making it worthy of involving more advanced techniques for future investigation to understand its actual incidence in cattle.

Apart from the difference in the cytogenetic technique employed so far for screening, the relative abundance of ROB than RCT in cattle compared to other species of animals including swine and humans might be attributed to the difference in the structure of chromosomes. In cattle, all the 29 chromosome pairs are acrocentric in morphology with one arm only and consequently centric fusion (ROB) could be more favourable to occur than in pigs and humans where one armed chromosomes make only a small part of the whole chromosomes karyotype (De Lorenzi *et al.*, 2012).

CYTOGENETIC TECHNIQUES FOR SCREENING CHROMOSOMAL ABNORMALITIES IN ANIMALS

Blood Cell Culture

Blood cell culture involves stimulating lymphocytes to grow by various lectins used as mitogens (Pokeweed, PHA, Concavalin A). Multiplying cells are stopped at metaphase using colcemid. The treatment of cells by hypotonic solutions enables to obtain well dispersed metaphase chromosomes for analysis using conventional Giemsa staining or banding techniques based on which karyotypes are constructed.

Conventional Method of Blood Cell Culture

Blood collected aseptically is the sample of choice for culture in domestic animals for screening chromosomal anomalies. About 1 mL of whole blood or buffy-coat layer separated by centrifuge can be used to initiate the culture. The culture media commonly consists of RPMI1640 (8 mL) foetal calf serum (2 mL), mitogen (e.g., Pokeweed mitogen, 0.1 mL), Antibiotics (0.1 mL Penicillin-Streptomycin) and L-glutamine (50 µL). In the case of whole blood culture, sodium heparin (50 µL) is also added to prevent coagulation. The cell culture is incubated for 72 hours at 37.5°C by agitating it once a day. Towards the end of the culture, colcemid (20 – 50 µL) is added for a period of 30 – 60 min to stop the cell cycle. The optimum concentration of colcemid and treatment plays an important role and hence, needs to be checked. To our experience, for bovine sample, a concentration of 40 µL for a treatment period of 30 min resulted suitable and elongated chromosomes. The culture is finally treated with a hypotonic solution (warm 0.075 M of KCL) for 20 min. After centrifuging the culture and removing the supernatant, the cells are fixed using Carnoy's fixative (3 part methanol, 1 part glacial acetic acid) added drop by drop. After exchanging the fixative three times by centrifuging and discarding the supernatant, the cell culture is left overnight at 4°C before making drops on slide and staining.

Early 5-bromo-2-deoxyuridine (BrdU) Incorporation in Conventional Cell Culture:

This involves addition of BrdU (15 µg/mL) to the cell cultures 8 h before harvesting. After 2.5 h, centrifuge the culture and remove the supernatant. The culture is restarted with complete fresh medium containing thymidine (10 µg/mL) for further 5.5 h. Ethidium bromide (5 µg/mL) 2 h before harvesting and 20 µL of colcemid 30 - 60 min before termination is added. For harvesting, the same protocol described for conventional blood culture above is followed. This technique provides good amount of 'pro-metaphase' plates suitable for G-banding and C-banding and is reported to offer better results in animals such as pig (Iannuzzi & De Berardino, 2008).

Synchronized Blood Culture

Thymidine is one of the commonly used chemical for culture synchronisation. For this purpose, a 200 µL thymidine (300 µg/mL of final concentration) is added to the 10 mL culture after 48 h of incubation. After 17 h of incubation with thymidine, the cell suspension is centrifuged and the supernatant is discarded. The cells are then washed with either fresh medium or with Hank's Balanced Salt Solution (HBSS), centrifuged at 1800 rpm for 8 min followed by removal of the supernatant. The culture is continued in a complete fresh medium that contains all the components, except the mitogen, for further 5.5 - 6 h. Two hours before the termination of the culture, 50 µL of ethidium bromide is also incorporated to prevent further contraction of chromosomes. Harvesting procedure including treatment using hypotonic solution and fixing cells is done the same way as described earlier for conventional culture. This procedure provides a high yield of late-prophase/early metaphase plates which are most suitable for high-resolution G- banding.

Conventional Giemsa Staining and Chromosome Banding Techniques

Giemsa Staining

The following day after the cells harvested from a conventional or synchronised blood culture, the cells pellet is re-suspended in a fresh fixative (0.5 to 1 mL depending on the quantity of the pellet). Drops of the cell suspension are made on slides, previously cleansed with ethanol and immersed in distilled water and kept at 4ºC. After drops are spreaded and dried in air, they are stained with 5% Giemsa solution in PBS for 3 min. Then, the slides are washed under running tap water and air-dried and examined under a microscope. Cultures with good mitotic index of metaphase or prometaphase chromosomes are identified and used for analyses of chromosome number, morphology and construction of conventional karyotypes. Conventional staining results in uniformly stained chromosomes that differ in size and shape. However, this method is not good enough to differentiate the chromosomes of different species like cattle and goats as they present similar shape and size. This is overcome by applying banding techniques based on elongated chromosomes obtained by reducing amount of colcemid added and treatment time (Iannuzzi & Beradino, 2008).

Most screening programmes for chromosome abnormalities which have been conducted in cattle especially in bulls intended for AI purposes used a simple Giemsa staining method. This has been applied mainly in European countries with the aim of controlling and eradication of ROB (1;29). However, the use of Giemsa staining technique, although it can detect ROB and chimerism for sex chromosome easily but it is less adequate to screen chromosome rearrangements like translocations of reciprocal type which involve the exchange of chromosome segments between non-homologous chromosomes. Hence, it is important to apply more advanced techniques such as high resolution G-banding (GTG) in order to avoid failure of detection and underestimation of chromosome abnormalities that cannot be detected using Giemsa as it is appeared to be for RCT in cattle.

Chromosome Banding Techniques

Chromosome banding is the process of eliciting differences in staining manifestation along the length of a chromosome thereby producing a specific banding pattern. These banding patterns are reproducible, distinctive, and are characteristics to each chromosome. They permit the identification of chromosomes, the construction of karyotypes, detailed description of structural rearrangements, and the comparison of chromosomes within a species or between closely or distantly related species (Hayes, 2000). Based on banding techniques, common chromosome banding nomenclature for bovids has been established (ISCNDB, 2000). The most common banding techniques include G-, R- and C-banding (Sumner, 1990).

GTG-Banding (G-banding by treatment of Trypsin and Giemsa staining)

G-banding involves digestion of less condensed chromatin regions by trypsin, thus reducing affinity to the Giemsa stain, resulting in G-negative bands. In contrast, highly condensed regions, which are less affected by trypsin, result in a more intense Giemsa stain (G-positive bands). There are several G-banding techniques. Slides prepared using elongated prometaphase chromosome spreads stored at room temperature are suitable for G-banding. Trypsin working solution is prepared fresh every time. The concentration of trypsin used might vary from 0.025% to 0.25% in PBS. In our laboratory, 0.25% offered consistent result for cattle. The procedure involves treatment of the slides first with 2xSSC solution in a water bath for 2 min at 60^{o}C. This is followed by dipping the slides in a freshly prepared trypsin solution for 2 min at room temperature. Immerse the slides in normal saline solution for 3 minutes and then, stain with 5% Giemsa in PBS for 10 minutes. Finally, the slides are rinsed with tap water and air dried prior to examination under a microscope for further morphological analysis and construction of karyotypes. The optimum time of treatment at each step should be checked. The procedure described here is modified from Gallimore and Richardson

(1973) and used in our laboratory (Yimer, 2011).

The banding technique such as high resolution G-banding in cattle has been used as a compliment technique for further detail information once abnormality is detected using Giemsa method and for establishment of standard chromosome karyotype (Iannuzzi, 1996). As it is costly and labour-some, its application for routine screening programme was limited in cattle. However, GTG has been used effectively for routine screening programmes for detection of RCT in pigs, in which RCT was found as the most common abnormality. The application of GTG in pigs is economically worthy considering the significant reduction of litter size in carriers of RCT. Moreover, GTG was applied in the establishment of standard normal chromosome nomenclature of a number of species of animals including goats (Iannuzzi *et al*., 1994) and buffalo (Iannuzzi *et al*., 1990).

Constitutive Heterochromatin (C-) Banding Technique

C-banding (constitutive heterochromatin banding) is the best technique for identifications of sex chromosomes and associated abnormalities as they present different pattern from autosomes. The technique involves the use of barium hydroxide to denature repetitive and non-repetitive DNA. When DNA is renatured with 2xSSC, the repetitive DNA sequences renature faster than the non-repetitive ones, visualizing centromeres made up by repetitive sequences.

Slides obtained from both normal and BrdU-treated cell cultures and stored at room temperature for two or more days can be used. Though there are different ways of C-banding, a procedure modified from Sumner (1972) is described in this paper and resulted repeatable result for cattle chromosomes in our laboratory (Yimer, 2011). Initially treat slides with 20% hydrogen peroxide (H_2O_2) in a water bath at 65^{o}C for 3 min. Then, rinse in normal saline at 65^{o}C for 1 min, followed by incubation in 5% $Ba(OH)_2$ solution at 65^{o}C for 3 min and rinsing under tap water. Following a rinse in distilled water and dipping in normal saline for 30 seconds, the slides are incubated in 2xSSC solution at 65^{o}C for 3 min. Finally, rinse the slides in mixture of normal saline and phosphate buffer and stain with 5% Giemsa for 20 minutes. After drying in air, the slides are examined under a microscope.

Fluorescent in Situ Hybridization (FISH) Technique

It is an advanced tool in the field of cytogenetics and has been applied as a classical technique in human cytogenetics. The technique is based on visualization of fluorescent hybridization signals present on specific chromosome regions (bands). This can be done by using DNA-probes containing fluorochromes conjugated with compounds that recognise the labelled DNA-probes. It is useful for precise identification of structural chromosome abnormalities and to study complex chromosome aberrations. Its application in animal cytogenetics is however limited by lack of commercial

availability of molecular probes unlike humans. Moreover, its application in animal cytogenetics for routine screening programme may not be economically worthy. A more detail description of the blood cell culture, banding techniques and FISH can be referred to the publication by Iannuzzi and Beradino (2008).

CONCLUSION

The importance of cytogenetic screening, as a part of a preventive measure for infertility problems associated with chromosome anomalies in a modern breeding system, has been very clear since Gustavsson has demonstrated the correlation between ROB (1;29) and reduced fertility in Swedish cattle in 1979. Abnormal chromosomal karyotypes lower reproductive performance of farm animals through decreasing ability or complete failure to produce functional gametes and death of embryos. Thus, it is quite important to extend cytogenetic investigations in farm animals including cattle breeds in the tropics to obtain a base line data on occurrence and significance of chromosomal anomalies that will lead to what strategies to be designed accordingly. Moreover, establishment of regulatory and monitoring systems to make sure animals (local or imported) intended for breeding are screened against cytogenetic anomalies would benefit the farming industry by minimizing associated reproductive failure. For more effective cytogenetic investigations, there is a need for integration among already established cytogenetic laboratories, more collaboration with breeders, veterinarians and animal scientists, as well as creating awareness among students leaving veterinary schools and animal health workers at farm level. Although the use of FISH technique with molecular markers offered great advantages for unambiguous identification of chromosomes and chromosome regions involved in chromosome abnormalities, its application is limited as it is costly and because of lack of commercial availability of chromosome specific probes. Nevertheless, the use of conventional Giemsa staining and banding techniques might be good enough to obtain repetitive and satisfactory results in cytogenetic investigations of common problems.

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Concentrations of Heavy Metals in Different Tissues of the Bivalve *Polymesoda erosa*: Its Potentials as a Biomonitor and Food Safety Concern

Yap, C. K.[1]*, Edward, F. B.[1] and Tan, S. G.[2]

[1]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Three populations of *Polymesoda erosa* collected from the west coast of Peninsular Malaysia were analyzed for heavy metals. Their soft tissues were dissected into muscle, foot, mantle, gill and remaining soft tissues (remainder). Overall metal concentrations (µg/g dry weight) in five soft tissues of the three clam populations were Cd (0.25-2.86), Cu (1.80-21.0), Ni (0.66-30.0), Pb (0.94-7.09), and Zn (79.2-365), and these were Cd (3.64-7.07), Cu (2.37-3.29), Ni (26.2-30.0), Pb (58.8-61.6), and Zn (3.84-8.78) for the shell ranges. Among the three *Polymesoda* populations, gill was found to have accumulated higher Cu and Zn concentrations compared to other soft tissues, whereas shell was found to have high levels of non-essential Cd, Pb and Ni. Information on heavy metals obtained in this study could serve as baseline data for this particular species since the information is lacking in the literature. The present study has evidently shown that different soft tissues of *P. erosa* are potential biomonitoring for Cd, Cu, Ni and Zn, whereas the clam shell as a potential biomonitoring material for Pb based on: (1) positive results based on biota-sediment accumulation factors (BSAF) (being macroconcentrators), (2) positive and significant correlations of metals between all five soft tissues (foot, gill, mantle, muscle and remainder) and the sedimentary geochemical fractions and total metal concentrations, and (3) comparisons to two similar burrowing bivalves (*Donax faba* and *Gelonia expansa*). Regardless of some metals in edible soft tissues having exceeded the food safety permissible limits, the concentrations of Cd, Cu, Ni and Zn in the soluble fractions (which is more bioavailable to consumers) of all the five edible soft tissues of *P. erosa* are below all the permissible metal limits. Therefore, these estimations clearly showed that the consumption of *P. erosa* could pose no toxicological risks to consumers.

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E-mail addresses:
yapckong@hotmail.com (Yap, C. K.),
sgtan@upm.edu.my (Tan, S. G.)
* Corresponding author

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INTRODUCTION

Elevated concentrations of heavy metals have been recorded in intertidal areas all over the world, which often reflect long-term pollution caused by human activities (Hedouin *et al*., 2009; Sakellari *et al.*, 2013; Zhao *et al.*, 2013). High degrees of industrialization and urbanization have led to strong risks of heavy metal contamination in the intertidal environment, particularly mangrove ecosystems in tropical countries (Lewis *et al.*, 2011; Wang *et al.*, 2013).

As a part of our continual biomonitoring effort following Mussel Watch in Malaysia (Yap, 2012) to assure that the pollution levels are controlled, this study focused on *Polymesoda erosa* (Corbicilidea family) which is a burrowing bivalve compared to the well-studied suspension-feeder green-lipped mussel *Perna viridis*. From the literature, this clam species is known to be hardy since they experience low pH, as indicated by the shape of the shells, which are badly eroded by acid mangal sediment (Morton, 1976). This clam species inhabits mangrove mudflats, which are under the constant influence of varying environmental stresses such as broad salinity range (7-22 ppt) (Ingole *et al.*, 1994) and it has been reported to possess high bioaccumulation capacity of heavy metals from ambient water (Modassir, 2000). Economically, it is an important, large and fleshy bivalve which can usually grow to marketable shell sizes ranging from 6 to 11 cm in length (Gimin *et al.*, 2004). In general, clams are good biomonitor of metal pollution (see Tarique *et al.*, 2013). Therefore, the above review generally supports the use of *P. erosa* as a potential biomonitor, besides being sedentary, long-lived, widely distributed in the mangrove area and the metal concentrations found in the soft tissues of bivalves can provide a time-integrated measurement of bioavailability of metals in the coastal waters (Phillips & Rainbow, 1993; Rainbow, 1995; Yap *et al.*, 2006).

The objectives of the present study were to: 1) provide concentrations of heavy metals in the different tissues of *P. erosa* from Malaysia; 2) assess its potential as a biomonitor for heavy metals; and 3) check whether the metal levels in the edible tissues of *P. erosa* exceeded some of the food safety permissible limits based on the total metals (total fractions) and soluble fractions in the edible soft tissues of the clams.

MATERIALS AND METHODS

About 25-30 of the bivalve *P. erosa* individuals from Telok Mas (Malacca), Sg. Sepang Kecil (Selangor) and Parit Jawa (Johore) (Fig.1), Peninsular Malaysia, were used for the metal analysis. However, surface sediment was only collected from the sampling site at Sg. Sepang Kecil due to the fact that the other two sites were actually bought from roadside stalls. In order to compare with the same burrowing bivalves, two different bivalves species, namely, *Donax faba* and *Gelonia expansa*, were collected from Pasir Panjang (Negeri Sembilan) and Kg. Pasir Puteh (Johore)

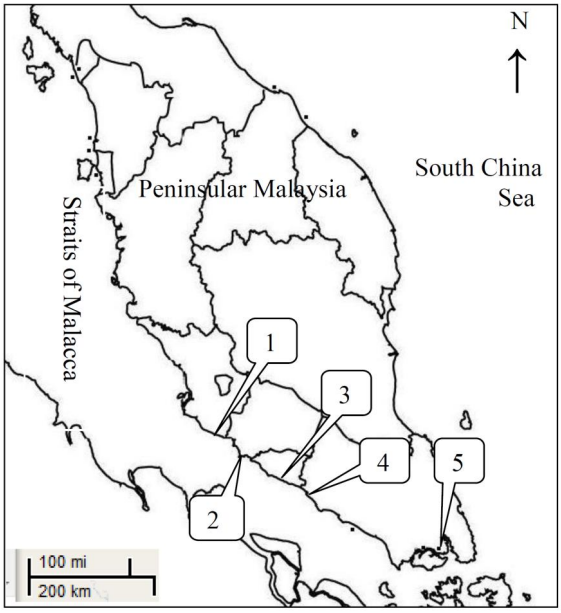

Fig.1: Map showing all the sampling sites of bivalves in Peninsular Malaysia. Note: 1= Sg. Sepang Kecil; 2= Pasir Panjang; 3= Telok Mas; 4= Parit Jawa; 5= Kg. Pasir Puteh.

(Fig.1), respectively, together with their habitat surface sediments. All the samples were collected between 28 and 30 April 2006. The identification of the bivalves was based on the book by Malaysia Fisheries Directory (2005) issued by the Department of Fisheries Malaysia. All bivalves were measured for shell lengths, shell heights, shell widths, total soft tissues wet weight, total soft tissues dry weight, condition index (CI) and water contents, and their values are presented in Table 1.

All the bivalve populations were dissected, divided and pooled into muscle, foot, mantle, gill and remaining soft tissues (remainder). The sediments, shells, and all the five different pooled categories of bivalve soft tissues were dried at 60°C in an oven to constant dry weights. Later, the dried sediments were grinded by using pestle and mortar, and sifted through a 63 µm stainless steel aperture.

Geochemical fractions of Cd, Cu, Ni, Pb and Zn in the sediments were obtained by using the modified sequential extraction technique (SET), as described by Badri and Aston (1983). Two replicates (N= 2) were obtained from the sieved main sample. The four fractions used in the present study were easily, freely, leachable or exchangeable (EFLE), acid-reducible (AR), oxidisable-organic (OO) and resistant fractions.

Triplicates of each dried category of the bivalve tissues were digested in concentrated HNO_3 (Analar grade, BDH 69%). The resistant fractions of the SET were digested in a combination of concentrated nitric acid (69%) and perchloric acid (60%) (ratio 4:1), as in the direct aqua-regia method. They were first placed in a hot-block digester first

at a low temperature for 1 hour and then fully digested at a high temperature (140°C) for at least 3 hours (Yap *et al.*, 2003a).

The digested samples were diluted to 40 mL with double-distilled water. After filtration, the concentrations of Cd, Cu, Ni, Pb and Zn were determined by using an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin-Elmer Model AAnalyst 800. All data are presented in μg/g dry weight.

In order to see the difference of metal bioaccumulation between two different size groups of *P. erosa*, the clams of a single population was divided into two groups of shell lengths: 60.0-66.1mm and 50.0-57.8mm. Total soft tissues of the two size groups were analyzed similarly for the five metals described previously.

In order to estimate the metal portion soluble or to make it more available to consumers, the soluble and insoluble metal fractions in the different soft tissues of the three *P. erosa* populations were determined, whereby the two fractions were separated by using a modified method as described by Bragigand *et al*. (2004). Ground dried soft tissues (0.5 g) were homogenized with 2mL of Ultra-turrax in a TRIS buffer solution 20nM, NaCl 150 mM, pH 8.6, at 4°C, based on 4 mL/g fresh weight, as suggested by Bragigand *et al*. (2004). The soluble and insoluble fractions were separated by centrifugation (25 rpm for 55 min). After centrifugation, S fraction was analyzed using AAS. The insoluble fraction was further digested with 2.5 mL of nitric acid (HNO_3) (1 mL of HNO_3 per 0.2 g of I). After digestion, acid solutions were made up to 10 mL with double distilled water (DDW), before being analyzed similarly for the five metals described previously.

To avoid possible contamination, all glassware and equipment used were acid-washed. Procedural blanks and quality control samples made from standard solutions for Cd, Cu, Ni, Pb and Zn were analyzed after every 5-10 samples in order to check for sample accuracy. Besides, the analytical procedures for the bivalves and soils were checked with the Certified Reference Material (CRM) for dogfish liver (DOLT-3, National Research Council Canada) and soil (International Atomic Energy Agency, Soil-5, Vienna, Austria). The recoveries of all the metals were satisfactory (see Table 2).

In this study, CI of the bivalves was determined because it is an indicator of the bivalve's physiological state and also as a guide to the bivalve's metabolic response to environmental stress (Yap *et al.*, 2002a). The CI (g/cm^3) value was calculated as the quotient of the total soft tissue dry weight for each bivalve and the shell volume (shell width [cm] × shell length [cm] × shell height [cm]) for each bivalve, multiplied by a constant, namely, 1000 (Lares & Orian, 1997; Yap *et al.*, 2003b). The formula is as follows:

$$\frac{\text{total soft tissue dry weight (g)}}{\text{Shell volume (cm}^3\text{)}} \times 1000$$

In order to estimate the distribution of metals occurred between the different tissues of bivalves and in associated

TABLE 1
Measurements (mean ± standard error) of shell lengths (SL; cm), shell heights (SH; cm), shell widths (SW; cm), total soft tissues wet weight (WW; g), total soft tissues dry weight (DW; g) condition index (CI; g/cm^3) and water contents (WC; %) of the three species of bivalves in the present study

Species	N	SL	SH	SW	WW	DW	CI	WC
Polymesoda								
Telok Mas	10	5.29 ± 0.06	4.71 ± 0.07	2.81 ± 0.04	6.83 ± 0.23	0.95 ± 0.04	13.6 ± 0.50	86.0 ± 0.62
SSK	10	6.28 ± 0.06	5.84 ± 0.05	3.35 ± 0.05	5.62 ± 0.28	0.81 ±0.04	6.57 ± 0.26	85.6 ± 0.39
Parit Jawa	5	5.50 ± 0.09	4.90 ± 0.12	3.19 ± 0.10	8.52 ± 0.89	1.32 ± 0.15	15.3 ± 1.14	84.4 ± 1.11
Donax faba	19	3.68 ± 0.15	2.64 ± 0.13	1.83 ± 0.11	2.59 ± 0.34	0.51 ± 0.07	26.0 ± 0.82	80.6 ± 0.36
Gelonia expansa	10	7.16 ± 0.33	6.71 ± 0.29	3.92 ± 0.18	12.5 ± 0.73	1.94 ± 0.18	9.32 ± 0.31	84.5 ± 0.59

Note: N= number of samples analyzed. SSK= Sungai Sepang Kecil

TABLE 2
Comparison of heavy metal concentrations between measured and certified values of certified reference material for Dogfish liver and soil. All values are presented in µg/g dry weight

Metal	Sample	Certified values (C)	Measured values (M)	Recovery (M/C × 100%)
Cd	Dogfish-liver	19.4 ± 0.600	20.5 ± 0.439	106 ± 2.26
	Soil-5	1.5	1.41	94.60
Cu	Dogfish-liver	31.2 ± 1.00	26.5 ± 2.58	85.0 ± 8.28
	Soil-5	77.1	72.9	94.4
Pb	Dogfish-liver	NA	NA	NA
	Soil-5	129	144.7	112.2
Ni	Dogfish-liver	2.72 ± 0.350	2.77 ± 0.741	102 ± 27.2
	Soil-5	13.0	12.3	94.6
Zn	Dogfish-liver	86.6 ± 2.40	80.9 ± 1.94	93.4 ± 2.24
	Soil-5	368	326	88.6

Note: CRM value is not available.

sediment, biota-sediment accumulation factors (BSAF) was calculated (based on a formula by Szefer *et al*., 1999; see below) for the five metals in the *P. erosa* from Sg. Sepang Kecil, *D. faba* and *G. expansa,* where their habitat surface sediments were collected and these three bivalves species are burrowers.

$$BSAF = \frac{Cx}{Cs}$$

Where C*x* and C*s* are the mean metal concentrations in the different tissues of bivalves and non-resistant fractions in the habitat surface sediment, respectively. In the present study, the non-resistant fractions (summation of EFLE, AR and OO) were used as sediment metal value in the BSAF calculation because the non-resistant is more related to its bioavailability characteristic to the living organisms and of anthropogenic sources (Yap & Wong, 2011).

For statistical analyses, comparison of metal levels of any two different size groups was performed by using t-test. On the other hand, Analysis of Variance (ANOVA) was applied to test the differences between the mean values of the metal concentrations with the subsequent comparison between individual means using Student-Newman-Kuels (S-N-K) multiple comparison test (Zar, 1999). The relationships between the different parts and sediment were tested using Pearson's correlation coefficient based on the concentrations of Cd, Cu, Ni, Pb and Zn. The correlation analysis was carried out by using log transformed data (Leung *et al*., 2005). All the above statistical analyses were performed by using SPSS version 12.

RESULTS AND DISCUSSION

The mean values of condition index (CI), water contents and other allometric parameters of *P. erosa* are shown in Table 1. The CI of the clams collected from Sg. Sepang Kecil, Parit Jawa and Telok Mas, was 6.57 ± 0.261 g/cm^3, 15.3 ± 1.14 g/cm^3 and 13.6 ± 0.50 g/cm^3, respectively. The percentages of water content were 85.6 ± 0.39%, 84.4 ± 1.11% and 86.0 ± 0.62% for Sg. Sepang Kecil, Parit Jawa and Telok Mas, respectively, and these are within the ranges reported for the oyster species [72.5-90.0%] (Watling & Watling, 1976), the blue mussel *Mytilus edulis* [85.8%] and *Perna viridis* [81.8-85.4%] (Yap *et al.*, 2003b).

i) Metal concentrations in the different tissues of bivalves

Heavy metal concentrations in the different tissues of *P. erosa* are shown in Table 3. The overall metal concentrations (µg/g dry weight) in the five soft tissues (foot, gill, mantle, muscle and remaining soft tissues) of three *Polymesoda* populations are Cd (0.25-2.86), Cu (1.80-21.0), Ni (0.66-30.0), Pb (0.94-7.09), and Zn (79.2-365), while for the shell ranges are Cd (3.64-7.07), Cu (2.37-3.29), Ni (26.2-30.0), Pb (58.8-61.6), and Zn (3.84-8.78).

When compared to soft tissues of *P. erosa*, the metal ranges (µg/g dry weight) for the two other burrowing bivalve species (*D. faba* and *G. expansa*) are broader and higher in Cd (0.30-3.92), Cu (4.71-79.8), Pb (0.95-39.7), and Zn (28.3-379), but these are narrower and lower in Ni (1.58-15.4). When compared to shells of *P. erosa*, the metal

TABLE 3
Concentrations (mean ± standard error, µg/g dry weight) of heavy metals in the different soft tissues of three populations of *Polymesoda erosa*, in comparison to those of *Donax faba* and *Gelonia expansa* populations

Species	Tissues	Cd	Cu	Ni	Pb	Zn
Polymesoda erosa						
Parit Jawa	Foot	1.47 ± 0.14^{ab}	3.77 ± 0.28^{a}	7.83 ± 0.45^{a}	2.18 ± 0.66^{a}	92.1 ± 11.4^{b}
	Gill	1.46 ± 0.21^{ab}	12.8 ± 0.19^{c}	11.1 ± 0.36^{b}	2.56 ± 0.29^{a}	263 ± 0.75^{c}
	Mantle	2.40 ± 0.11^{c}	9.34 ± 0.60^{b}	15.1 ± 0.67^{c}	6.44 ± 0.35^{b}	270 ± 1.14^{c}
	Muscle	1.79 ± 0.08^{b}	3.58 ± 0.23^{a}	7.76 ± 0.20^{a}	3.11 ± 0.72^{a}	104 ± 9.88^{b}
	Remainder	1.17 ± 0.09^{a}	11.8 ± 0.49^{c}	6.22 ± 0.36^{a}	2.07 ± 0.61^{a}	79.2 ± 4.17^{b}
	Shell	7.07 ± 0.22^{d}	3.17 ± 0.36^{a}	28.9 ± 0.40^{d}	61.6 ± 1.67^{c}	6.47 ± 0.16^{a}
Sg. Sepang Kecil	Foot	0.48 ± 0.11^{a}	3.36 ± 0.53^{a}	3.98 ± 0.24^{a}	2.10 ± 0.40^{a}	87.5 ± 14.1^{b}
	Gill	1.22 ± 0.00^{bc}	21.0 ± 0.00^{e}	23.5 ± 0.00^{c}	4.61 ± 0.00^{a}	349 ± 0.00^{c}
	Mantle	1.36 ± 0.00^{c}	13.2 ± 0.00^{c}	12.6 ± 0.00^{b}	4.79 ± 0.00^{a}	365 ± 0.00^{c}
	Muscle	0.78 ± 0.10^{ab}	5.76 ± 0.13^{b}	5.28 ± 0.07^{a}	3.41 ± 0.16^{a}	150 ± 9.14^{b}
	Remainder	0.73 ± 0.10^{ab}	14.9 ± 0.69^{d}	5.26 ± 0.32^{a}	4.35 ± 0.34^{a}	154 ± 32.4^{b}
	Shell	3.64 ± 0.12^{d}	2.37 ± 0.30^{a}	30.0 ± 0.44^{d}	58.8 ± 1.47^{b}	3.84 ± 0.77^{a}
Telok Mas	Foot	0.25 ± 0.02^{a}	1.80 ± 0.23^{a}	0.66 ± 0.15^{a}	0.94 ± 0.25^{a}	105 ± 0.53^{b}
	Gill	2.86 ± 0.00^{c}	8.81 ± 0.00^{c}	10.7 ± 0.00^{c}	6.51 ± 0.00^{c}	263 ± 0.00^{d}
	Mantle	1.64 ± 0.30^{b}	5.82 ± 0.23^{b}	11.8 ± 0.04^{d}	7.09 ± 0.25^{c}	264 ± 2.30^{d}
	Muscle	0.70 ± 0.08^{a}	1.86 ± 0.12^{a}	2.61 ± 0.34^{b}	1.89 ± 0.53^{ab}	129 ± 2.27^{c}
	Remainder	0.61 ± 0.11^{a}	8.60 ± 0.87^{c}	1.87 ± 0.49^{ab}	3.59 ± 0.62^{b}	138 ± 8.28^{c}
	Shell	6.60 ± 0.18^{d}	3.29 ± 0.40^{a}	26.2 ± 0.36^{e}	59.6 ± 0.72^{d}	8.78 ± 1.27^{a}
Donax faba						
Pantai Pasir Panjang	Foot	3.68 ± 0.14^{a}	5.98 ± 0.39^{a}	1.58 ± 0.09^{a}	2.36 ± 0.08^{c}	38.8 ± 0.66^{c}
	Gill	3.92 ± 0.18^{a}	12.9 ± 0.54^{c}	4.76 ± 0.15^{c}	2.09 ± 0.08^{c}	93.4 ± 1.16^{e}
	Mantle	2.95 ± 0.14^{a}	6.86 ± 0.10^{a}	3.25 ± 0.58^{b}	1.47 ± 0.10^{b}	46.8 ± 2.29^{d}
	Muscle	3.74 ± 0.35^{a}	4.71 ± 0.20^{a}	2.03 ± 0.44^{ab}	2.51 ± 0.13^{c}	28.3 ± 0.81^{b}
	Remainder	2.74 ± 0.17^{a}	15.1 ± 1.32^{d}	2.70 ± 0.33^{ab}	0.95 ± 0.16^{a}	42.6 ± 0.45^{cd}
	Shell	7.20 ± 0.43^{b}	9.88 ± 0.16^{b}	28.5 ± 0.19^{d}	5.27 ± 0.17^{d}	4.56 ± 0.01^{a}
Gelonia expansa						
Kg. Pasir Puteh	Foot	0.36 ± 0.09^{a}	79.8 ± 32.6^{a}	9.47 ± 0.18^{a}	19.4 ± 2.53^{b}	124 ± 3.56^{b}
	Gill	1.21 ± 0.00^{b}	26.2 ± 0.00^{a}	15.4 ± 0.00^{c}	19.5 ± 0.00^{b}	379 ± 0.00^{e}
	Mantle	0.71 ± 0.17^{ab}	61.6 ± 1.92^{a}	11.4 ± 0.05^{b}	39.7 ± 0.93^{c}	275 ± 1.42^{d}
	Muscle	0.30 ± 0.03^{a}	7.73 ± 2.67^{a}	10.4 ± 0.65^{ab}	4.53 ± 0.91^{a}	214 ± 7.01^{c}
	Remainder	0.59 ± 0.16^{ab}	19.7 ± 0.71^{a}	8.58 ± 0.40^{a}	4.72 ± 1.07^{a}	180 ± 34.6^{c}
	Shell	6.99 ± 0.29^{c}	2.80 ± 0.26^{a}	25.0 ± 0.48^{d}	56.9 ± 0.65^{d}	6.67 ± 0.30^{a}

Note: Values sharing similar alphabet are not significant different with others, $P > 0.05$.

ranges (μg/g dry weight) for the other two burrowing bivalve species are narrower but slightly higher in Cd (6.99-7.20), broader, and also higher in Cu (2.80-9.88), almost within the Ni range (25.0-28.5), broader but slightly lower in Pb (5.27-56.6), and within the Zn range (4.56-6.67). However, it is difficult to assess whether *P. erosa* is a good biomonitor for heavy metals based on the above comparisons. The following section (ii) on the correlation analysis and BSAF values could better assess the potential of *P. erosa* as a biomonitor.

Higher metal concentrations found in the different tissues of *P. erosa* from Sg. Sepang Kecil indicated a high metal contamination at the sampling site. This indication was complemented by the lower condition index (CI) values found in the Sg. Sepang Kecil samples than two other locations. This also suggested that *P. erosa* from Sg. Sepang Kecil could have suffered from metal pollution stress, since the different tissues of these clam exhibited the highest concentrations (μg/g dry weight) of Cu in the remainder (14.9 ± 0.69) and gill (21.0); and Zn in the mantle (365) and gill (349). The negative relationships between the bivalves' CI values and metal concentrations in the mussel soft tissues have been reported by Yap *et al.* (2002a). Therefore, it is assumed that the higher metal levels and lower CI values in *P. erosa* collected from Sg. Sepang Kecil have a higher metal bioavailabilities and contamination at the sampling site. The higher Cu and Zn levels found in some tissues of *P. erosa* collected from Sg. Sepang Kecil could be explained by the domination of non-resistant fractions of Cu (63.9%; Yap *et al.*, 2013) and Zn (55.1%; Yap *et al.*, 2011) in the surface sediments, indicating anthropogenic sources (Yap & Pang, 2011).

Furthermore, the high metal concentrations in the clams indicated high metal bioavailabilities of metals at the sampling site. This is because analysis of bivalves can provide measurement of the integrated bioavailability of metals in coastal environment (Phillips & Rainbow, 1993; Rainbow, 1995; Silva *et al.*, 2001; Rainbow *et al.*, 2002; Yap *et al.*, 2006).

The shells of *P. erosa* are the best accumulators of non-essential Cd, Ni and Pb. The shells of *P. erosa* from Parit Jawa exhibited the highest concentrations of Cd (7.07 ± 0.216 μg/g dry weight) and Pb (61.6 ± 1.67 μg/g dry weight), indicating Cd and Pb of water and sediment as sources of these metals in Parit Jawa. The assumption could be made because bivalve shells have been used quite frequently as biomonitoring materials for heavy metals (see Yap *et al.*, 2003c; Lazaretha *et al.*, 2003; Gillikin *et al.*, 2005; Protasowicki *et al.*, 2008).

Fig.2 shows the heavy metal concentrations in the two different size groups of bivalve *P. erosa*. Smaller sized groups (50.0-57.8mm) accumulated higher concentrations of nonessential Cd, Ni and Pb (although not significantly different for Cd and Ni; $p>0.05$) when compared with the larger sized groups (60.0-66.1mm). This is in accordance with the results reported by Gilek *et al.* (1996) and Yap *et al.* (2003d) that smaller bivalves accumulate greater

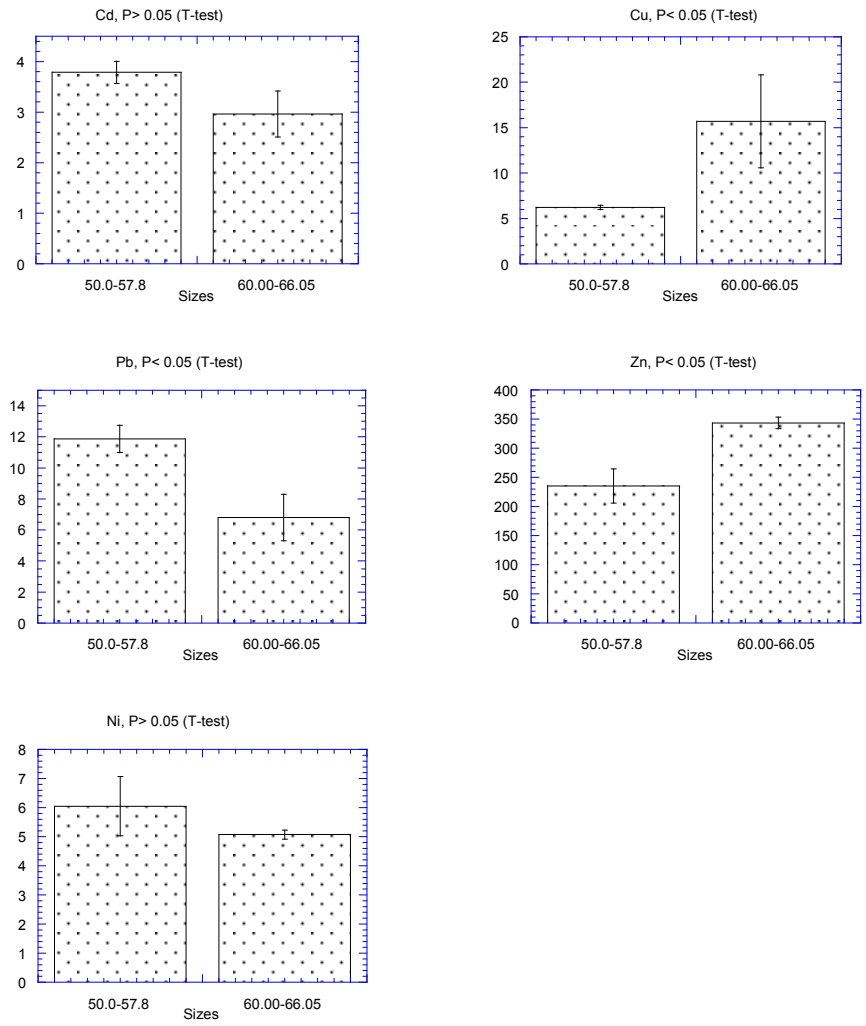

Fig.2: Comparisons of concentrations (mean ± standard error, µg/g dry weight) of heavy metals [nonessential (Cd, Ni and Pb) and essential (Cu and Zn)] between two significant different sized groups (shell lengths: 60.0-66.1mm and 50.0-57.8mm) of *Polymesoda erosa.*

concentrations of heavy metal. According to Gilek *et al.* (1996), tissue concentrations decreased with increasing body size, and the size-dependent dissimilarities in bioaccumulation were caused primarily by size-related differences in uptake rate. However, larger individual accumulated more concentrations of Cu and Zn when compared with the smaller individuals ($p<0.05$). This could be due to higher metabolic rates probably possessed by the larger individual may be resulting in higher accumulation rates of Cu and Zn, since these two metals are essential to the living organisms. Also, this could be explained by the differences in the accumulation

pathways and the processes affecting the bioavailability of the nonessential metals to *P. erosa* are dissimilar to essential metals (Yap *et al.*, 2003d).

The gill and the remainder of *P. erosa* accumulated higher concentrations of Cu when compared to mantle and the other soft tissues. Meanwhile, the mantle and the gill accumulated higher concentrations of Zn than other tissues (Fig.3). The metal distribution in the different tissues of bivalves could be related to cellular processes participated in metal metabolisms (Marigómez *et al*., 2002). For example, the higher metal levels found the gills could be explained by the gill being a key interface for dissolved metal uptake (Marigómez *et al.*, 2002). Other explanations are differences in affinities of metals to bind to metallothionein in the different tissues where they incorporated into lysosomes and later being eliminated towards the circulating hemocytes and blood plasma of bivalves (Roesijadi, 1980). The reason for this was the high (or low) concentrations of metal found in the different tissues could be related to the different rates of accumulation and excretion of metals due to internal metal handling (Gundacker, 1999).

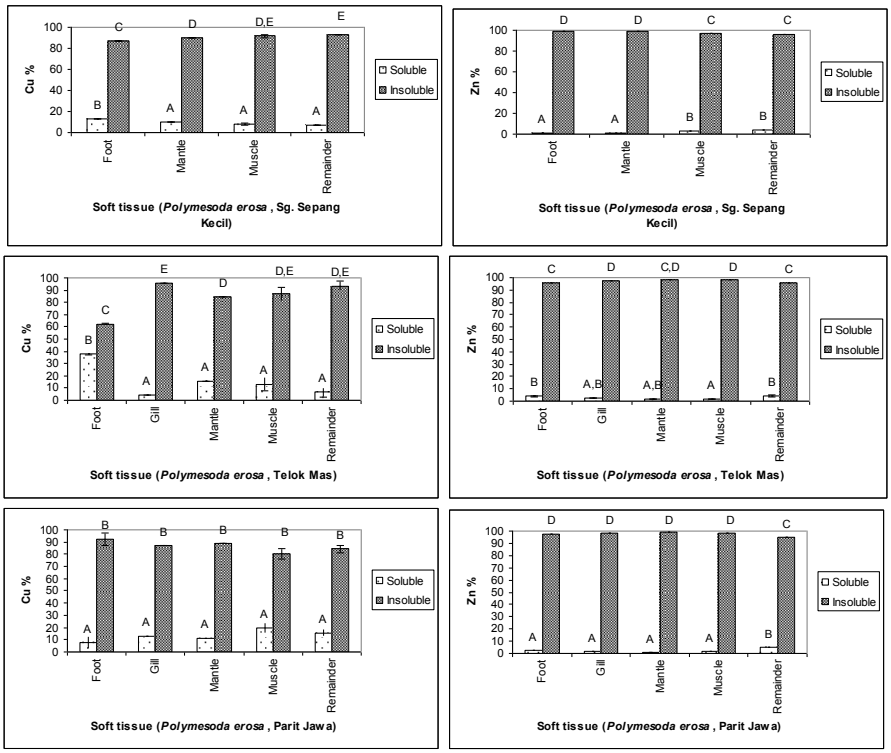

Fig.3: The soluble and insoluble fractions (%) of essential metals (Cu and Zn) in the different soft tissues of the *Polymesoda erosa*.

Note: Values sharing similar alphabet are not significant different with others, P > 0.05.

ii) Assessment of the potential of Polymesoda as a biomonitor for heavy metals

The potential of *P. erosa* as a biomonitor of the five metals are based on: (1) correlation coefficients of heavy metal concentrations between the different tissues of three burrowing bivalves species and their geochemical fractions of surface sediment (see Table 4); (2) BSAFs based on the different parts of the three bivalves species (see Table 5), and (3) comparison to two similar burrowing bivalve species (*D. faba* and *G. expansa*) based on the ground of points 1 and 2 above.

BSAF was calculated for all the different tissues of bivalves with the assumption that the distribution of a metal in the environment is controlled by a continuous exchange among phrases such sediment and molluscs (Hsu *et al.*, 2006). BSAF is used to classify the different tissues of bivalves as macroconcentrators (BSAF> 2), microconcentrators (1< BSAF< 2) and deconcentrators (BSAF < 1), as proposed by Dallinger (1993).

For *Polymesoda* clams (Table 4), all the five soft tissues (foot, gill, mantle, muscle, and remainder) positively and significantly correlated (R= 0.752-0.997; P< 0.001) with AR, OO and SUM, while foot, muscle and shell only weakly and positively correlated (P< 0.05) with EFLE. From Table 5, all the six soft tissues of *P. erosa* appear to be macroconcentrators (> 2.0) for Cd, five soft tissues (foot, gill, mantle, muscle and remainder) as macroconcentrators for Zn, three tissues (gill, mantle and remainder) as macroconcentrators for Cu, and three tissues (gill, mantle and shell) as macroconcentrators for Ni. Only shell appears to be macroconcentrator for Pb. Since these tissues are also significantly correlated with the sedimentary geochemical fractions and total metal concentrations, therefore, *P. erosa* is a potential biomonitor of Cd, Zn, Cu and Ni.

For *D. faba* (Table 4), four tissues (foot, gill, mantle and muscle) only weakly and positively correlated (P< 0.05) with OO, resistant and SUM. Only shell strongly corrected (R= 0.779; P< 0.001) with EFLE. From Table 5, only shells of *D. faba* appear to be macroconcentrator for Cd and Ni, three tissues (gill, remainder and shell) as macroconcentrators for Cu and four tissues (foot, gill, mantle and remainder) for Zn. Three tissues are found to be microconcentrators (1<BCF<2) for Cd and Cu, while only two tissues as microconcentrator. Others are found to be deconcentrators (BCF < 1). Therefore, together with the correlation results in Table 4, only the shells of *D. faba* could be evidently shown as potential biomonitoring materials for Cd and Ni, while soft tissues for Zn and Cu require further studies.

For *G. expansa* (Table 4), all the five soft tissues positively and significantly correlated (R= 0.599-0.983; at least P< 0.05) with all the four fractions (EFLE, AR, OO and resistant) and SUM. From Table 5, only the shells of *G. expansa* appear to be macroconcentrator for Cd and Pb, while two tissues as macroconcentrators for Cu and Zn. Gill is found to be microconcentrators

TABLE 4
Pearson's correlation of five metals (Cd, Cu, Ni, Pb and Zn) concentrations between the different tissues of three burrowing bivalves species and their geochemical fractions of surface sediment. N= 15.

Species	Different parts	EFLE	AR	OO	Resistant	SUM
Polymesoda erosa	Foot	0.524*	0.962**	0.974**	0.431	0.779**
	Gill	0.414	0.868**	0.997**	0.406	0.752**
	Mantle	0.451	0.929**	0.994**	0.403	0.759**
	Muscle	0.529*	0.964**	0.976**	0.457	0.797**
	Remainder	0.456	0.908**	0.967**	0.477	0.794**
	Shell	0.586*	-0.225	-0.312	0.567*	0.272
Donax faba	Foot	-0.580*	-0.246	0.535*	0.577*	0.546*
	Gill	-0.380	-0.235	0.616*	0.572*	0.558*
	Mantle	-0.392	-0.206	0.627*	0.564*	0.555*
	Muscle	-0.553*	-0.180	0.573*	0.577*	0.558*
	Remainder	-0.417	-0.444	0.449	0.463	0.426
	Shell	0.779**	0.169	-0.044	-0.389	-0.310
Gelonia expansa	Foot	0.639*	0.599*	0.938**	0.877**	0.927**
	Gill	0.901**	0.869**	0.868**	0.909**	0.963**
	Mantle	0.743**	0.695**	0.957**	0.954**	0.983**
	Muscle	0.939**	0.924**	0.744**	0.799**	0.875**
	Remainder	0.861**	0.853**	0.795**	0.802**	0.888**
	Shell	-0.078	-0.174	0.094	0.142	0.049

Note: ** Correlation is significant at the 0.01 level (2-tailed);* Correlation is significant at the 0.05 level (2-tailed); EFLE= Easily, freely, leachable. extractable; AR=Acid-reducible; OO=Organic oxidisable; SUM= Summation of EFLE, AR and OO.
The *Polymesoda* clams were collected from Sg. Sepang Kecil.

(1<BCF<2) for Cu, and only two tissues (gill and shell) as microconcentrator for Ni. Only mantle was found to be microconcentrator for Pb while three tissues (foot, muscle and remainder) were found to be microconcentrator for Zn. Others were shown to be deconcentrators. Therefore, together with the correlation results in Table 4, only the shells of *G. expansa* could be evidently shown as potential biomonitors for Cd and Pb, while soft tissues for Zn and Cu; however, further studies are still needed.

Therefore, *P. erosa* appears to be the best biomonitor of heavy metals when compared to *D. faba* and *G. expansa*. Although the findings of the present study indicate potentials of *P. erosa* as a good biomonitor of heavy metal pollution, other recommended criteria for a good biomonitor should be tested, including genetic variations of different geographical populations (Yap *et al.*, 2002b) and its metal accumulation capacity under laboratory experimental studies (Yap *et al.*, 2004), before it can be used as a valid biomonitor.

TABLE 5
Biota-sediment accumulation factors (BSAF) based on the different parts of bivalves. Calculation of BSAFs based on metal concentrations in the different dissected tissues of bivalves and non-resistant fractions in the sediment.

Species	Different parts	Cd	Cu	Ni	Pb	Zn
Polymesoda erosa	Foot	2.78	1.14	1.21	0.55	3.12
	Gill	6.61	6.59	6.56	1.17	12.15
	Mantle	7.37	4.42	3.47	1.21	12.85
	Muscle	4.33	1.96	1.61	0.89	5.38
	Remainder	4.00	5.06	1.60	1.13	5.54
	Shell	20.67	0.81	9.10	15.28	0.14
Donax faba	Foot	1.23	1.71	0.13	0.25	2.46
	Gill	1.16	3.69	0.29	0.24	4.39
	Mantle	0.98	1.96	0.27	0.16	2.90
	Muscle	1.25	1.35	0.17	0.27	1.80
	Remainder	0.91	4.32	0.22	0.10	2.78
	Shell	2.40	2.83	2.35	0.56	0.29
Gelonia expansa	Foot	0.32	3.36	0.71	0.78	1.01
	Gill	0.95	1.07	1.05	0.76	3.05
	Mantle	0.62	2.59	0.86	1.59	2.24
	Muscle	0.25	0.33	0.78	0.18	1.74
	Remainder	0.48	0.83	0.64	0.19	1.47
	Shell	5.92	0.12	1.87	2.28	0.05

Note: *Polymesoda* clams were collected from Sg. Sepang Kecil.

iii) Estimation of metal soluble fractions and comparisons with the food permissible limits

The soluble and insoluble fractions (%) of essential metals (Cu and Zn) in the different edible soft tissues of *P. erosa* are presented in Fig.3, while those of nonessential metals (Cd, Ni and Pb) are given in Fig.4. In general, the insoluble fractions dominated (> 50%) in the five metals investigated. This could be due to the fact that the metals were generally and strictly bound to metallothionein (Viarengo *et al*., 1985; Roesijadi, 1992). In addition, the formation of a metal-thiolate complex with the cysteine residues inside the lysosomes has caused the slower release of heavy metals found in the different edible soft tissues (Yap *et al*., 2003e), which could result in high percentages of insoluble fractions. Also, the high percentages in the insoluble fractions could be due to the soft tissues being linked to mineral granules (Geffard *et al*., 2002), which could limit the elimination of heavy metals from the edible soft tissues.

For the essential Cu and Zn (Fig.3), the soluble fractions in all the edible soft tissues of *P. erosa* ranged from 4.40-38.20% (13.11 ± 2.24%) and 0.60-5.10% (2.45 ± 0.36%), respectively, whereas for the nonessential

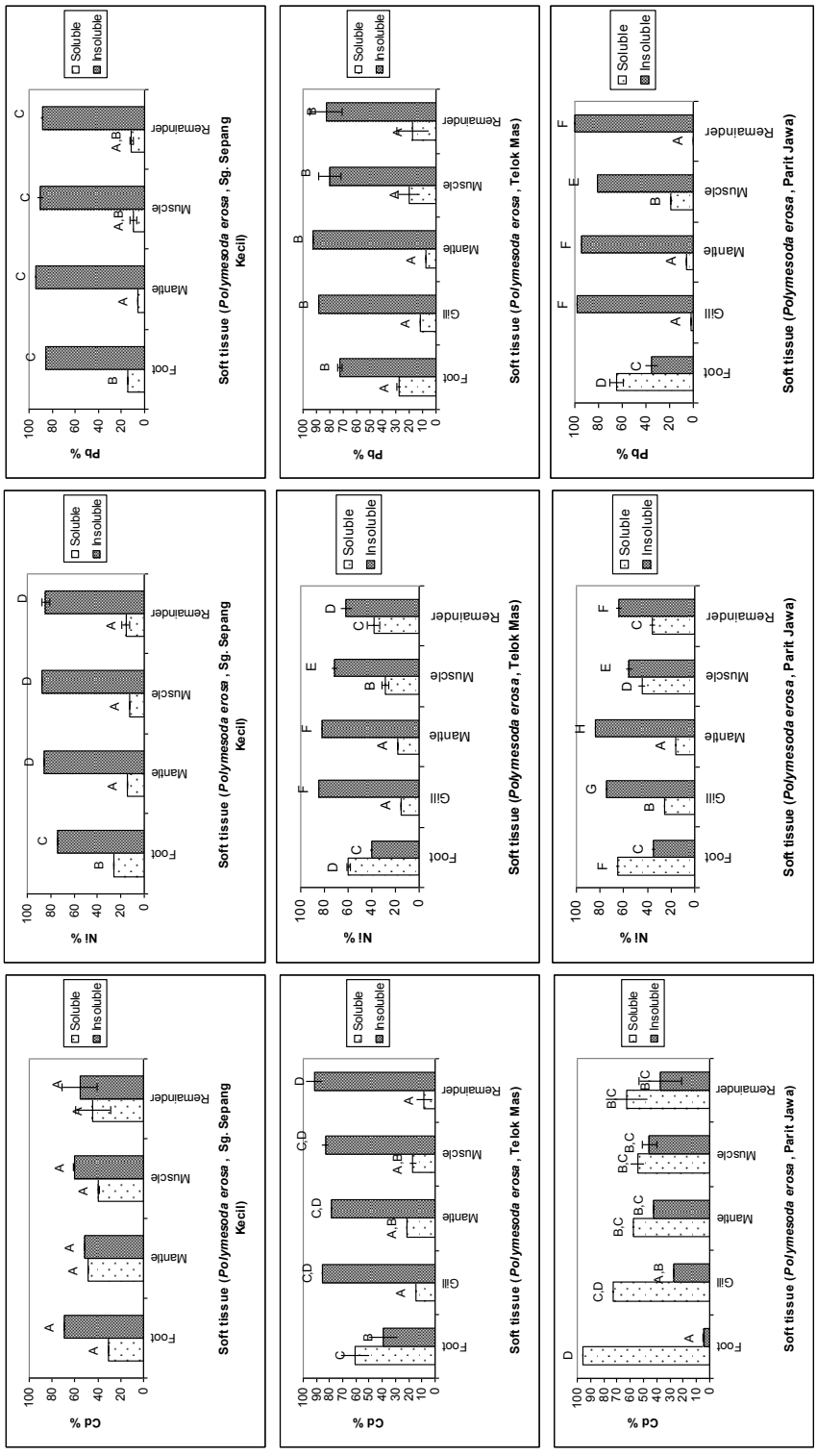

Fig.4: The soluble and insoluble fractions (%) of nonessential metals (Cd, Ni and Pb) in the different soft tissues of the *Polymesoda erosa*. Note: Values sharing similar alphabet are not significant different with others, $P > 0.05$.

Cd, Ni and Pb (Fig.4), the soluble fractions ranged from 8.40-95.40% (44.76 ± 6.62%), 12.40-65.30% (29.68 ± 4.58%) and 4.40-64.40% (15.57 ± 4.25%), respectively. This clearly indicated that the essential metals have lower percentages of soluble fractions when compared to the nonessential metals. This indicated that the essential metals could be possibly and tightly bound to metallothionein and therefore significantly higher percentages of insoluble fractions of Cu and Zn were found. However, this interesting discovery disagrees with the finding by Yap *et al.* (2012) in which they found the essential Zn was not tightly bound to metallothionein and thus easily remobilized (Yap *et al.*, 2012) after boiling. Since the finding by Yap *et al.* (2012) was highly supported by previous studies (see Phillips, 1985; Yap *et al.*, 2002c), and the differences of metal fractionations in both studies, the present results need further studies in the future.

The estimation of the total quantities of metals (total fraction) present in edible soft tissues of bivalves will lead to an overestimation of the quantities likely to be bioavailable to a consumer (Bragigand *et al*., 2004). Therefore, the determination of the soluble and insoluble fractions employed in the present study was significant. This is because the information on the soluble fractions in the bivalves is important in order to estimate the toxicological risks of the bivalves to their consumers. It is assumed that the metals present in the soluble fractions are more bioavailable to consumers than metals bound to insoluble fractions (Wallace *et al*., 2003). Nevertheless, according to Bragigand *et al*. (2004), the physic-chemical characteristics of a heavy metal in the soft tissues also play a significant role in determining the bioavailability of dietary metal in the digestive tract of the consumer. As suggested by the finding of Geffard *et al*. (2002) in the *Crassostrea gigas*, the higher the level of contamination, the more important metal-binding to cytosolic ligands for Cd will be. This could explain the elevated metal percentages found in the soluble fractions of the foot and mantle of the bivalves, in which the locations of the tissues are more exposed to the contaminated external environment.

The levels of Cd, Cu, Pb and Zn found in the total and soluble fractions of the edible tissues of *P. erosa* were compared with some established food safety guidelines or maximum permissible limits set by some countries or agencies (Table 6). No permissible limits for Ni could be found, and therefore, no comparison was made for the levels of Ni in the bivalves.

From Table 6, the total fractions of Cd and Pb in gill and mantle of *P. erosa* are both below all the permissible limits, except for the metal limits set by USEPA (2003) for Median International Standards for metals in mollusks. However, the total fraction of Pb in mantle is slightly higher than that by the Ministry of Public Health, Thailand (MPHT, 1986). All total fractions of Zn in all the five edible soft tissues exceeded the metal limits by USEPA (2003). In addition, total fractions of gill and mantle also exceeded the Maximum permissible levels

TABLE 6
Comparisons of heavy metal concentrations (µg/g dry weight) in the different edible soft tissues of *Polymesoda erosa* based on total metal concentrations (total fraction) and soluble fractions, with some selected food permissible limits (also presented in µg/g dry weight).

		Cd	Cu	Pb	Zn
Foot	Total fraction	0.25-1.47	1.80-3.77	0.94-2.18	87.5-105
	Soluble fraction	0.30-0.49	0.15-1.05	0.87-1.56	0.99-3.16
Gill	Total fraction	1.22-2.86	8.81-21.0	2.56-6.51	263-349
	Soluble fraction	0.41-0.89	0.35-1.29	0.16-0.88	3.59-3.81
Mantle	Total fraction	1.36-2.40	5.82-13.2	4.79-7.09	264-365
	Soluble fraction	0.33-0.75	0.72-1.05	0.51-0.86	1.83-3.32
Muscle	Total fraction	0.70-1.79	1.86-5.76	1.89-3.41	104-150
	Soluble fraction	0.17-0.35	0.33-0.68	0.82-1.01	1.54-3.91
Remainder	Total fraction	0.61-1.17	8.60-14.9	2.07-4.35	79.2-154
	Soluble fraction	0.08-0.44	0.40-1.27	0.01-1.13	4.23-5.8
1. Permissible limit set by the ministry of Public Health, Thailand (MPHT, 1986)		NA	133	6.67	667
2. Australian Legal Requirement (NHMRC, 1987)		10.0	350	NA	750
3. Maximum permissible levels established by Brazilian Ministry of Health (ABIA, 1991)		5.0	150	10	250
4. Food and Drug Administration of the USA (USFDA, 1990)		25.0	NA	11.5	NA
5. Median International Standards for metals in mollusks compiled by the Food and Agricultural Organization of the United Nations (USEPA, 2003)		2.00	10-30	1.00-6.00	40-100

Note: NA= Not available.
All the ranges for total and soluble fractions above are based on three populations of *P. erosa* as shown in Table 3 except for soluble fraction of gill which is only based on two populations from Telok Mas and Parit Jawa.

established by Brazilian Ministry of Health (ABIA, 1991). It is good to see that all the total fractions of Cu in the different edible soft tissues are below all the permissible limits. Regardless of some metals in the edible soft tissues having exceeded the food safety permissible limits, all the four metal levels in the soluble fractions of all the five edible soft tissues of *P. erosa* are below all the permissible metal limits. Therefore, this estimation clearly shows that the consumption of *P. erosa* could pose no toxicological risks to the consumers.

CONCLUSION

From the present study, the metal levels found in the different soft tissues and shells of three populations of *P. erosa* are important for future reference. Being macroconcentrators based on BSAFs, and positively and significantly correlations of metals between all the five soft tissues (foot,

gill, mantle, muscle and remainder) and the sedimentary geochemical fractions and total metal concentrations, the different soft tissues of *P. erosa* are potential biomonitoring tissues for Cd, Zn, Cu and Ni, while the clam shell is a potential biomonitoring material for Pb. In comparison to two similar burrowing bivalves (*D. faba* and *G. expansa*), *P. erosa* was shown to be the best biomonitor of heavy metals. Regardless of some metals in the edible soft tissues having exceeded the food safety permissible limits, all the four metal levels in the soluble fractions of all the five edible soft tissues of *P. erosa* are below all the permissible metal limits. Therefore, these estimations clearly showed that the consumption of *P. erosa* could pose no toxicological risks to the predators (consumers).

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Correlation and Path Analyses of Seed Yield in Okra (*Abelmoschus esculentus* (L.) Moench) Grown Under Different Cropping Seasons

Adekoya, M. A.*, Ariyo, O. J., Kehinde, O. B. and Adegbite, A. E.

Institute of Agricultural Research and Training, Obafemi Awolowo University, P.M.B. 5029, Moor Plantation, Ibadan, Nigeria

ABSTRACT

Inter-character correlations and path coefficient analyses of yield related characters were evaluated in some quantitative traits of okra (*Abelmoschus esculentus* (L.) Moench) grown under different cropping seasons. Data collected were subjected to correlation and path coefficient analyses to determine the relationships among the characters, as well as the direct and indirect effects of some yield related characters to seed yield. Correlation coefficients among characters varied among seasons. Days to flowering, number of pods per plant, length of matured pod, weight of matured pods per plant, number of ridges per pod, number of seeds per pod and 100 seed weight had significant genotypic correlations with seed yield/plant across the seasons. Environmental correlation coefficients were generally low and non significant except for number of leaves per plant, length of matured pod, width of matured pod and weight of matured pods per plant in all the seasons of study. The genotypic correlation coefficients of eight selected characters with seed yield were partitioned into their direct and indirect effects. Weight of matured pods per plant had the largest direct effect on seed yield/plant in early rain 2005 (season 1) and off season 2006 (season 2), branch length in the early rain 2006 (season 3) and stem height at flowering in late rain 2006 (season 4). The residual effects of 0.16 in season 1, 0.74 in season 2, 0.55 in season 3 and 0.38 in season 4 respectively accounting for 84%, 26%, 45% and 62%, contributed by the characters under study.

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E-mail addresses:
munawarm11@yahoo.com (Adekoya, M. A.),
ariyojo@yahoo.com (Ariyo, O. J.),
tundekehinde04@yahoo.com (Kehinde, O. B.),
gokeadegbite@yahoo.co.uk (Adegbite, A. E.)
* Corresponding author

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INTRODUCTION

In tropical agriculture, production and conservation of okra is imperative due to the

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usefulness of its young leaves and immature pods for human food and animal feed. In Nigeria, okra (*Abelmoschus esculentus* (L.) Moench) is widely grown during both the dry and rainy seasons. The rainy season production is bimodal, where its early cropping usually begins as early as March/April and ends during a short dry spell in August, whilst the late cropping starts from August to October/November. The meteorological data, including rainfall and temperature, vary during the early and late seasons in ways that influence growth and yield of crop (Olasantan & Bello, 2004). The dry season cultivation is done in the dry month of the year spanning from December to March in swamps. Testing for the development of high performing genotypes in different environments is a continuous exercise to increase local production which in turn helps to boost the income of farmers.

Season of production is a parameter identified to be borne in mind in order to obtain maximum yields in okra (Adekoya, 2008). A number of okra varieties grown in Nigeria have low yield due to associated biotic and abiotic factors, lack of improvement, limited agro-inputs and low quality of seeds among others (Adekoya, 2008). Before yield improvement can be ascertained, appropriate selection criteria must be well established. One method to achieve this is to determine the extent of relationship between any given pair of traits (Obisesan, 1985; Adekoya, 2008; Adebisi, 2008). In addition, Falconer (1981) suggested that a plant breeder should also know whether the improvement of one character will result in simultaneous change in the other characters through estimates of inter-character correlation.

The use of simple correlation analysis cannot fully explain the relationships among characters. Therefore, path coefficient analysis has been used by many researchers for a more complete determination of the impact of an independent variable on a dependent one (Akinyele & Osekita, 2006; Lal, 2007; Zhao *et al*., 2008, Karademir *et al*., 2009; Majid *et al*., 2011; Udensi & Ikpeme, 2012; in okra, fennel, wheat, cotton, potato and *Cajanus cajan*, respectively). The path coefficient analysis helps the breeder to explain direct and indirect effects which have been extensively used in breeding works of different crop species by various researchers (Punia & Gill, 1994; Shalini *et al*., 2000; Ali *et al*., 2002; in rapeseed and sunflower). The knowledge of inter-character relationships is very important in plant breeding for indirect selection for characters that are not easily measured and for those that exhibit low heritability.

A path coefficient analysis simultaneously captures the effects of intricate relationship among various traits under investigation. Information obtained from correlation coefficients can be enhanced by partitioning them into direct and indirect effects for a set of a priori cause–effects interrelationship, as has been demonstrated in various crops (Kang *et al*., 1983; Gravois & Helms, 1992; Gravois & McNew, 1993; Board *et al*., 1997; Murtadha *et al*., 2004). However, there has

been relatively little information on some of the newly collected genotypes of okra in Nigeria. A careful study of relationships among quantitative characters is necessary in order to ascertain the magnitude and direction of changes to be expected during selection in okra. This study was therefore designed to identify agronomic characters that determine the seed yield in okra using correlation and path coefficient analyses in twenty genotypes of okra grown under four different cropping seasons.

MATERIALS AND METHODS

Twenty genotypes of okra, *Abelmoschus esculentus* (L.) Moench were sown in four different seasons [early rain (last week in July) 2005, off season (second week in January), early rain (first week in June) and late rain (second week in September), 2006)]. The genotypes were obtained from different sources as listed in Table 1. These include four established genotypes which had been released to farmers and sixteen others that were still under evaluation for yield and stability. After ploughing and harrowing, the experiment was laid out in a randomized complete block design (RCBD) with three replications and in single row plot to minimize variation. A block consisted 20 rows of all the genotypes and each row was 4.5m long. The rows were 0.75m apart, while the between plant distance in each row was 0.45m, thus each row contained eleven plants.

Three weeks after sowing, plants were thinned to one plant per hole. From nine inner plants in each row, data were collected on the following characters:
days to flowering which was determined as the average of the number of days to flowering of the nine inner plants in each row; plant height at flowering was measured from the soil level to the tip of the plant and number of pod per plant, which was the average value of summation of pods from nine inner plants in each row. Number of leaves per plant (determined by counting the number of nodes on the main stem and all the branches from the inner plants in each row), while number of branches per plant was the number of branches on the main stem while branch length was determined as the average value of the summation of length of branches from nine inner plants.

Final plant height was taken by measuring the plant from the soil level to the tip of the main stem when the plants had shed their leaves and other floral parts and the shoots had dried up, length of matured pod was determined when pods turned brown and fibrous on the stem while width of matured pod was measured as the circumference of the matured harvested pods. Weight of matured pods per plant was taken as average value of the summation of the weighed matured harvested pods from nine inner plants. Meanwhile, number of ridges per pod was determined at maturity by counting the number of the ridges in ten randomly selected pods, number of seeds per pod was determined at maturity by counting the number of seeds in ten randomly selected pods, 100–seed weight was determined by weighing 100 dry seeds as sample from the bulk of each genotype

and seed yield per plant was determined by bulking the yield of the dry seeds of nine inner plants and dividing by nine.

Data obtained were analyzed using SAS statistical package (SAS, 1999). Genotypic and phenotypic correlations were calculated from the mean values of the genotypes using the procedure outlined by Miller *et al.* (1958) as reported by Ariyo (1995). Environmental correlation coefficients were calculated according to the procedure of Falconer (1981). Path analysis to estimate the direct and indirect contributions of some characters to seed yield/plant was also conducted using the method described by Dewey and Lu (1959), as reported by Ariyo (1995). Fig.1 shows the cause and effect system between seed yield/plant and eight agronomic characters.

TABLE 1
Genotypes, sources and status of the accessions used for the study

Serial number	Genotypes	Sources	Status
1	NHAe 47-4	NIHORT	ESTABLISHED
2	NHAe 47- 4-5	NIHORT	ENTRANT
3	CLEMSON SPINELESS	NACGRAB	ESTABLISHED
4	LADY'S FINGER	UNAAB	ENTRANT
5	LADY'S FINGER (OUTCAST)	UNAAB	ENTRANT
6	LD 88/1 – 8 – 11 – 1	NIHORT	ENTRANT
7	NHAe88/82	NACGRAB	ENTRANT
8	NHAe99/28	NACGRAB	ENTRANT
9	NHAe99/DA	NACGRAB	ENTRANT
10	NIHORT ILAGIDI	UNAAB	ENTRANT
11	OK 20	NIHORT	ENTRANT
12	OLA 3 LOCAL	NIHORT	ENTRANT
13	OLA99/13	NIHORT	ENTRANT
14	OLA K 2005	NIHORT	ENTRANT
15	OLA KA 1 – 6 – 05	NIHORT	ENTRANT
16	OLA V_1	NIHORT	ENTRANT
17	OSADEP: PURPLE TALL	UNAAB	ENTRANT
18	SHORT MOUTH IBARAPA	UNAAB	ENTRANT
19	V_2 – OYO	UNAAB	ESTABLISHED
20	V – 35	IAR&T	ESTABLISHED

Status: Established – Accessions already released to farmers
Entrant – Accessions still undergoing evaluation on the field (not yet released to farmers)

NIHORT : National Institute of Horticultural Research and Training
NACGRAB : National Centre for Genetic Resources and Biotechnology
UNAAB : University of Agriculture, Abeokuta
IAR&T : Institute of Agricultural Research and Training

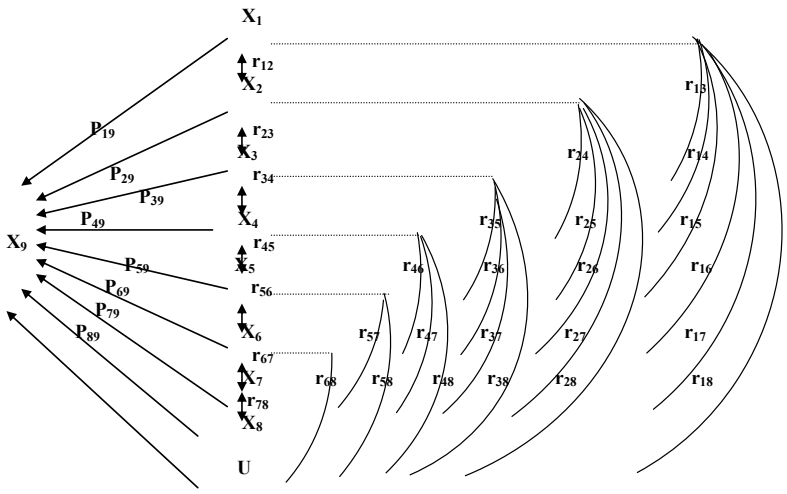

Fig.1: Causation diagram indicating the relationships between seed yield and yield related traits.

RESULTS

The genotypic correlation coefficients among sixteen characters in okra in four seasons are presented in Table 2. In early rain, 2005 (season 1), seed yield/plant exhibited significant positive genotypic correlations with all traits studied, except length of fresh pod, width of fresh pod, length of matured pod and 100 seed weight. In off season, 2006 (season 2), significant positive genotypic correlations were recorded between seed yield/plant and most of the characters evaluated except stem height at flowering, length of fresh pod and length of matured pod. In early rain, 2006 (season 3), seed yield/plant displayed significant positive genotypic correlations with all traits studied except length of fresh pod while in late rain, 2006 (season 4), it exhibited positive significant correlation with most characters except length and width of fresh pod, number of branches/plant and 100 seed weight.

From Table 3, the differences in both magnitude and direction of correlation coefficients were observed in the four different seasons for some of the characters. Positive significant correlation coefficients were observed for most traits except for the length of fresh pod in all seasons, 100 seed weight in seasons 1 and 4, and stem height at flowering and length of matured pod in season 2.

Environmental correlation coefficients between seed yield/plant and other agronomic characters in okra in four seasons are presented in Table 4. In all seasons, correlation coefficients ranged between 0.01 and 1 in the two directions. Negative significant correlation were recorded between seed yield/plant and number of branches/plant in season 1, length and width of matured pod in season 2, days to flowering, stem height at flowering, length of fresh pod, length and width of matured pod and number of ridges/pod in season 4.

TABLE 2
Genotypic correlation coefficients between seed yield per plant and other agronomic characters in okra in four seasons

Agronomic Characters	seed yield per plant			
	season 1	season 2	season 3	season 4
Days to flowering	1.23**	-0.09	0.94**	-0.08
Stem height at flowering	0.54**	-0.42**	0.92**	0.70**
Length of fresh pod	-0.47**	-0.44**	-0.43**	-0.62**
Width of fresh pod	-0.06	0.07	0.08	-0.41**
Number of pods /plant	0.89**	1.40**	1.14**	1.13**
Number of leaves/ plant	0.71**	-0.06	0.85**	0.02
Number of branches/ plant	0.26*	0.17	1.00**	-0.34**
Branch length	0.72**	-0.18	1.15**	0.85**
Final plant height	0.51**	0.25*	0.98**	0.60**
Length of matured pod	-0.35**	-0.67**	-0.06	-0.1
Width of matured pod	0.26*	0.52**	0.71**	0.46**
Weight of matured pods/plant	0.91**	0.72**	0.94**	1.12**
Number of ridges/pod	1.01**	0.27*	1.27**	0.68**
Number of seeds/pod	0.85**	4.83**	0.09	0.62**
100 seed weight	-0.69**	0.42**	0.75**	-0.54**

*, ** = significant at 5% and 1% level of probability respectively
n= 60, Degree of freedom = n-2=58

TABLE 3
Phenotypic correlation coefficients between seed yield per plant and other agronomic characters in okra in four seasons

Agronomic Characters	seed yield per plant			
	season 1	season 2	season 3	season 4
Days to flowering	0.76**	-0.08	0.49**	-0.14
Stem height at flowering	0.36**	-0.29**	0.55**	0.55**
Length of fresh pod	-0.29*	-0.25*	-0.25*	-0.60**
Width of fresh pod	-0.22	-0.13	0.09	0.21
Number of pods /plant	0.95**	0.53**	0.96**	0.90**
Number of leaves/ plant	0.56**	0.05	0.70**	0.25*
Number of branches/ plant	0.01	0.07	0.69**	0.33**
Branch length	0.57**	-0.13	0.78**	0.69**
Final plant height	0.36**	0.09	0.66**	0.54**
Length of matured pod	-0.09	-0.50**	0.24	-0.2
Width of matured pod	0.26*	0.29*	0.53**	0.26*
Weight of matured pods/plant	0.95**	0.58**	0.96**	0.97**
Number of ridges/pod	0.68**	0.19	0.52**	0.29*
Number of seeds/pod	0.83**	-0.03	0.41**	0.53**
100 seed weight	-0.36**	0.33**	0.49**	-0.36**

*, ** = significant at 5% and 1% level of probability respectively
n= 60, Degree of freedom = n-2=58

TABLE 4
Environmental correlation coefficients between seed yield per plant and other agronomic characters in okra in four seasons

Agronomic Characters	seed yield per plant			
	season 1	season 2	season 3	season 4
Days to flowering	0.02	-0.08	0.01	-0.76**
Stem height at flowering	-0.07	-0.07	0.29*	-0.57**
Length of fresh pod	-0.01	0.29*	-0.09	-0.52**
Width of fresh pod	-0.18	0.22	-0.08	0.62**
Number of pods /plant	1.01**	0.36**	0.92**	-0.1
Number of leaves/ plant	0.38**	0.31*	0.66**	0.83**
Number of branches/ plant	-0.27*	-0.04	0.53**	0.45**
Branch length	0.37**	-0.06	0.56**	0.32*
Final plant height	0.09	-0.23	0.48**	0.21
Length of matured pod	0.79**	-0.35**	0.55**	-0.82**
Width of matured pod	0.37**	-0.31*	0.47**	-1.10**
Weight of matured pods/plant	0.99**	0.44**	0.97**	0.45**
Number of ridges/pod	0.04	0.1	-0.02	-1.27**
Number of seeds/pod	0.81**	0.09	0.46**	0.23
100 seed weight	0.21	0.21	0.33**	0.25*

*, ** = significant at 5% and 1% level of probability respectively
n= 60, Degree of freedom = n-2=58

The direct and indirect path coefficients that estimated the extent of the relationships between seed yield/plant and eight yield related characters using the genotypic correlation values are presented in Table 5. Of the sixteen traits evaluated in this study, eight were genotypically significantly correlated with seed yield in the two directions. Path analysis partitioned these correlation coefficients into their components, thus giving an insight to which traits can be selected for improvement in seed yield. Number of pod/plant and final plant height had significant correlation coefficients in all seasons and large indirect effect through the branch length but had negative direct effects. This indicated that improvement in seed yield can be made indirect selection for branch length.

DISCUSSION

Phenotypic correlation is a composite of genotypic and environmental correlations. In this study, it was observed that the genotypic correlation coefficients were, in most cases, higher than their corresponding phenotypic correlation coefficients indicating that the inherent association between the characters is governed largely by genetic causes, although it could also be affected by environmental forces. Similar finding were earlier observed by Ariyo *et al*. (1987) and Scod *et al*. (1995) in okra.

The significant positive genotypic correlations between seed yield/plant and number of branches per plant in all the cropping seasons may be related to greater photosynthetic capacity provided by more leaves. Also more branches imply more

TABLE 5
Direct and indirect effect of some characters on seed yield in okra

Character	Environment	Direct effect	Days to flowering	Plant height at flowering	Number of pods per plant	Branch length	Final plant height	Width of matured pod	Weight of matured pods/plant	100 seed weight	Genotypic correlation coefficient
Days to flowering	1	0.06		-0.02	0.13	0.34	-0.01	0.11	0.60	0.02	1.23**
	2	-0.32		-0.07	0.15	0.16	-0.62	0.66	-0.41	0.36	-0.09
	3	0.57		0.06	-0.17	0.37	-0.55	0.12	0.67	-0.13	0.94**
	4	-0.79		0.37	-0.15	-0.86	0.80	0.02	-0.05	0.58	-0.08
Plant height at flowering	1	-0.11	0.01		0.06	0.28	-0.03	0.09	0.25	-0.01	0.54**
	2	-0.57	-0.04		0.70	0.19	-0.15	0.07	-0.56	-0.05	-0.42**
	3	0.26	0.13		-0.16	1.03	-1.04	0.11	0.81	-0.23	0.92**
	4	6.20	-0.05		-0.84	-2.55	-3.77	-0.05	1.05	0.70	0.70**
Number of pods per plant	1	0.19	0.04	-0.04		0.24	-0.04	-0.01	0.43	0.08	0.89**
	2	-0.56	0.09	0.71		-0.25	0.51	0.89	0.70	-0.68	1.40**
	3	-0.17	0.59	0.25		1.91	-1.77	0.25	0.73	-0.65	1.14**
	4	-1.10	-0.11	4.71		-2.62	-2.29	0.40	1.85	0.29	1.13**
Branch length	1	0.37	0.06	-0.09	0.12		-0.07	0.04	0.22	0.06	0.72**
	2	0.34	-0.15	-0.32	0.42		-0.43	0.34	-0.27	-0.10	-0.18
	3	1.41	0.15	0.19	-0.23		-1.16	0.10	0.91	-0.23	1.15**
	4	-2.40	-0.28	6.57	-1.20		-3.69	0.19	1.19	0.48	0.86**
Final plant height	1	-0.11	0.01	-0.03	0.07	0.22		0.07	0.29	0.01	0.51**
	2	-0.91	-0.22	-0.10	0.31	0.16		0.78	-0.10	0.32	0.25*
	3	-1.38	0.23	0.20	-0.21	1.19		0.11	0.96	-0.11	0.98**
	4	-4.01	0.16	5.83	-0.63	-2.21		-0.14	0.86	0.74	0.60**
Width of matured pod	1	0.16	0.04	-0.07	-0.02	0.10	-0.05		0.17	-0.08	0.26*
	2	1.23	-0.17	-0.03	-0.40	0.09	-0.57		0.25	0.13	0.52**
	3	0.34	0.21	0.08	-0.12	0.41	-0.46		0.79	-0.55	0.71**
	4	0.60	-0.02	-0.50	-0.73	-0.74	0.92		0.97	-0.04	0.46**
Weight of matured pods/ plant	1	0.59	0.06	-0.05	0.14	0.14	-0.05	0.05		0.04	0.91**
	2	1.37	0.10	0.24	-0.29	-0.07	0.06	0.22		-0.91	0.72**
	3	0.75	0.52	0.28	-0.16	1.72	-1.77	0.36		-0.76	0.94**
	4	1.59	0.02	4.09	-1.28	-1.80	-2.17	0.37		0.29	1.12**
100 seed weight	1	-0.08	-0.01	-0.01	-0.18	-0.29	0.01	0.16	-0.28		-0.69**
	2	-0.87	0.13	-0.03	-0.44	0.04	0.34	-0.18	1.44		0.42*
	3	-0.67	0.11	0.09	-0.16	0.48	-0.22	0.28	0.84		0.75**
	4	-0.74	0.61	-5.83	0.43	1.56	4.01	0.04	-0.62		-0.54**

Residual effect: Early rain 2005 (season 1) = -0.16; Dry season 2006 (season 2) = 0.74; Early rain 2006 (season 3) = -0.55; late rain 2006 (season 4) = -0.38

*, ** Significant at 5% and 1% level of probability, respectively

fruit bearing nodes. The positive significant association of days to flowering with seed yield in early rain 2005, early rain and late rain 2006 indicated that early flowering lines were favoured by these environments. This may be due partly to the considerable decline in rainfall relatively in these environments, a situation that placed the early flowering lines at a relative advantage in terms of productivity.

Significant genotypic and phenotypic correlations of seed yield per plant with plant height at flowering, length of fresh pod, number of pods per plant, width of matured pod, weight of matured pods per plant and 100-seed weight in all the seasons imply that these characters possessed greater practical values for selection. Negative correlations of seed weight per plant with length of fresh pod suggests that selection for okra genotypes with long pod will give low seed yield probably because of physiological ability of the plant to successfully feed higher numbers in short pods. In early rain and late rain 2006, significant genotypic and phenotypic correlations of number of pods per plant, number of branches per plant and number of ridges per pod with seed yield suggested that selection based on phenotypic correlations would be effective. The significant genotypic and phenotypic correlations between number of ridges per pod and number of seeds per pod with seed weight suggested that there would be more seed yield since the greater the number of ridges and seeds, the more the number of seeds per pod and hence the more the seed yield. Thus, selection for these traits will lead to improvement in okra seed. Similar reports were observed by Murtadha *et al*. (2004).

Characters which are phenotypically correlated but not genotypically correlated will not produce repeatable estimates of inter-character associations and any selection based on the relationship is likely to be unreliable. This is true of the relationship between width of matured pod and 100-seed weight in the late rain 2006.

Very few of the environmental correlation coefficients were significant in the four seasons and this showed that the effect of environmental factors on the expression of the relationship between the characters was not so strong as to alter it markedly. Significant environmental correlations were observed for seed yield with weight of matured pods per plant, width of matured pod, length of matured pod and number of pod per plant but these were also genotypically and phenotypically correlated in all the seasons of the study.

Correlation measures mutual association with no regard to causation, whereas path analysis specifies causes and measures their relative importance (Dewey & Lu, 1959). Plant height at flowering had the largest positive direct effect on seed yield with its largest indirect effect via weight of matured pods per plant, though its correlation with seed yield was significant. Despite the strong positive association between the number of pods per plant and seed yield, its direct effect was negative indicating the inefficiency of selection based on correlation alone. The final plant height,

width of matured pod and weight of matured pod per plant can be used for direct selection to improve seed yield in okra. Final plant height had a negative contribution with seed yield despite its significant correlation. This demonstrates the defects of selecting on the basis of inter-correlation as such a selection may not produce the desired results.

Weight of 100 seeds manifested high negative direct effect on seed yield. Its high positive indirect effects via days to flowering, branch length, final plant height and weight of matured pods per plant appeared to be the cause of their strong correlation with seed yield.

The residual effect of 0.16 in early rain 2005, 0.74 in off season 2006, 0.55 in early rain 2006 and 0.38 in late rain 2006 implied that 84%, 26%, 45% and 62% respectively of the total variation in seed yield had been determined. It further portrayed the influence of environment in the determination of genetic parameters.

In conclusion, the above findings illustrated that final plant's height, branch length and number of pod per plant were the most important component for higher seed yield. It was observed that the performance of the genotypes varied from season to season. Genotypes generally performed best in early rain followed by late rain which was as a result of favourable environmental conditions (lower rainfall, low relative humidity and higher temperature). It shows the early rainy season is the best growing period for okra.

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Light and Scanning Electron Microscopy of the Small Intestine of Young Malaysian Village Chicken and Commercial Broiler

Khalid K. Kadhim[1], Md Zuki Abu Bakar[1]*, Mohamed Mustapha Noordin[2], Mohd Amin Babjee[2] and Mohd Zamri Saad[2]

[1]Department of Veterinary Preclinical Sciences, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

The intestinal mucosa of the Malaysian village chicken (MVC) and commercial broiler breed (CBC) from day 1 to 20 post-hatch was examined by means of light and scanning electron microscopy. The results showed that the intestinal mucosa was highly developed on day 1 in CBC as compared to MVC in regard to villus height, crypt depth and thickness of tunica muscularis. At day 1 post-hatch, it was observed that the mid cecum of the CBC showed plicae with short villi, while very short villi without plicae were observed in MVC. The intestinal villi in both breeds had a finger-like shape at day-old chick and changed to plate-like and tongue-like shapes toward the end of the experiment for CBC and MVC, respectively. Unlike MVC, the surface epithelia of the intestinal villi in CBC exhibited cell activities represented by surface recesses, clear cell outline and dome-shaped cells with protuberances, while a marked corrugated surface and areas of discontinuities were dominantly seen on the intestinal villi of the MVC particularly on the ileal villi. This study revealed that the intestinal mucosa of MVC of newly hatched chick was less evolutionary as compared to CBC which showed earlier development and maturation with more active villi to provide the highest rate of absorption as the breed selected for high body weight and rapid growth rate.

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E-mail addresses:
khalidkamkad@yahoo.com (Khalid K. Kadhim),
zuki@upm.edu.my (Md Zuki Abu Bakar),
noordinmm@upm.edu.my (Mohamed Mustapha Noordin),
sm_amin@upm.edu.my (Mohd Amin Babjee),
mzamri@upm.edu.my (Mohd Zamri Saad)
* Corresponding author

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INTRODUCTION

The present Malaysian village chicken, commonly known as *ayam kampong*, are the result of cross-breeding between the

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Red jungle fowl and mixed exotic domestic breeds brought in by Europeans, mainly the British (Azahan & Zahari, 1983). Because broilers are marketed much earlier than in the past, many previous studies concentrated on the development of the gastro-intestinal tract (GIT) during the post-hatching growth period to illustrate the changes occurring in this tract during selection for fast growth (Bayer *et al.,* 1975; Yamauchi & Isshiki, 1991; Yamauchi *et al.,* 1992; Yamauchi, 2002).

The avian intestine lacks the macroscopic level of folding seen in large mammals (King & McLelland, 1979; Turk, 1982). Gussekloo (2006) established basic histological data on the intestinal villi of chickens. The mucosal surface area of the small intestine in chicken undergoes important changes during development which may determine the capacity to absorb nutrients (James *et al.,* 1988; Smith *et al.,* 1990). Mathan *et al*. (1976) and Calvert and Potheir (1990) studied the intestinal crypts at early postnatal period in rats and mice, and found that the crypts developed from the flat intervillus epithelium. At hatching, the villi of the small intestine are undeveloped while crypts in the intervillus spaces are not detectable (Geyra *et al*., 2001). The development of intestinal mucosa after hatching occurs in the duodenum and jejunum, but this is found to be lesser in the ileum of poult (Uni *et al*., 1999). The thickness of muscularis externa of the intestine is greater in heavy lines than in light lines (Ogiolda *et al.,* 1998). The development of cecal mucosa decreases from the base to apex of the caecum in chicken (Rezaian & Hamedi, 2007). The villi at the distal part of cecum represent a typical appearance accompanied with regression of the epithelium and glands (Kitagawa *et al*., 1996; Looper & Looper, 2005).

The SEM observations of the intestinal mucosa of different birds have been done by many authors, Yamauchi and Isshiki (1991) and Yamauchi (2002) who studied the mucous membrane of the intestine exhibiting regional variations and reflecting the changing functions of the digestive system. Other authors have studied the developmental aspects of intestinal villi in post-hatched chicks with the SEM (Bayer *et al.,* 1975; Yamauchi *et al*., 1992). The morphological changes in intestinal villus surface and villus tip related to intestinal function of chicken at different ages were observed by Shamoto *et al.* (1999), Samanya and Yamauchi (2001) and Maneewan and Yamauchi (2003). The tips of duodenal villi have more developed epithelial cell protuberances, cell cluster and cell exfoliation resulting in a rough surface in heavy breed compared to the smooth surface in the light breed on 1 and 10 days post-hatch (Yamauchi & Isshiki, 1991; Yamauchi, 2002). The comparisons of the epithelial cells of intestinal villi at first day and10 days post-hatched in heavy and light breed revealed that the intestinal villi of the heavy breed reached morphological and physiological maturation earlier than those in light breed (Yamauch *et al.,* 1992; Yamauchi, 2002).

In the present study, MVC, which is characterized by a slow growth rate, was compared with CBC as a breed selected for high growth rate. The experiment was undertaken to determine the alteration in the intestinal mucosa between these two breeds using light and scanning electron microscopy. The comparisons were made at 1, 10 and 20 days after hatching.

MATERIALS AND METHODS

Animals

A total of 35 birds of MVC and CBC were used in this study. The eggs of MVC were obtained from Jenderam Hulu, Sepang. The eggs were incubated and hatched in the laboratory. The day-old chicks of commercial line (Ross) selected for high body weight were supplied by a private hatchery (Linggi Poultry farm Sdn. Bhd. C-P lot 1354, Kuala Kangsar, Perak, Malaysia). The birds were reared in separate cages with food and water provided *ad libitum*.

Collection of Tissue Samples

Seven birds from each breed were serially euthanased at days 1, 10, and 20 post-hatch by intravenous (cutaneous ulnar vein) administration of sodium pentobarbitone (80mg/ kg) (Mitchell & Smith, 1991). The entire intestine was removed (duodenum, jejunum, ileum and cecum). The small intestine was then divided into duodenum, jejunum and ileum, following the demarcation set by Mitchell and Smith (1990) and Geyra *et al.* (2001). The organs were washed with saline solution to remove the intestinal contents.

Light Microscope Examinations

Sample from each segment was taken for histological examination which included the midpoint of each part of intestine (duodenum, jejunum, ileum and cecum). Specimens were fixed in 10% Neutral Buffered Formalin (NBF) for 24 hrs and processed according to the standard histological procedure. Sections of 3 µm thickness were cut and stained with Hematoxyline and Eosin, and Masson trichrome (Bancroft & Gamble, 2005). The sections were viewed under image analyzer (Olympus Image Analysis, BX 51 TF) provided by the CC12 camera.

Scanning Electron Microscope Examinations

One-millimetre slice of tissue from the middle portion of the duodenum, jejunum and ileum were fixed in 4% gluteraldyhyde. Tissue samples for SEM were processed as described previously (Yamauchi *et al*., 1990; Maneewan & Yamauchi, 2003). Specimens were mounted under the dissecting microscope to select the right orientation. The specimens were dried in a critical point drying apparatus (BALTEC-SPD 030) using liquid carbon dioxide as the medium, sputter-coated with gold (BALTEC-SCD 005 vacuum coater) at 100 millitorr, 7 milliamperes for 3 min, before they were examined with a Jeol- SEM (JSM-6400, Japan) at 8 kv.

RESULTS

Light Microscopic Observations

The histological observations revealed that the intestinal wall has multilayered tube containing four layers from the lumen to the external layer, the mucosa (tunica mucosa), the tunica submucosa, the tunica muscularis externa, which are represented by the inner thick circular and the outer thin longitudinal muscle fibres, and the serosa (tunica serosa). The tunica mucosa is folded into many villi. The mucosal epithelium consisted of four types of cells, chief cells, goblet cells, paneth cells and endocrine cells. These observations were similar for both breeds. However, the mucosa of small intestine of day-old chicks in CBC was found to be more developed than in MVC (see Fig.1). The differences represented by well develops villi and the crypt glands. These crypts of day-old MVC contained few cells, and invagination was not completed in all the intestinal segments particularly in the ileum. Furthermore, the thickness of tunica muscularis externa was higher in CBC than in MVC. However, the villi height, crypt depth and thickness of tunica muscularis externa were found to have increased thereafter.

The body of cecum showed similar histological layers like those of the intestine. However, in CBC, day-old chicks showed plicae (folds) with short villi, but areas without villi were also seen in the same section. A thin layer of longitudinal smooth muscle fibres represents the muscularis mucosa was also present. In MVC, very short villi without plicae were observed in the cecum of day-old chicks with additional shallow crypts that opened directly into the flat mucosal surface (Fig.2). The thin cecum wall had relatively very thin circular smooth muscle layer as compared to CBC. In 10-day-old MVC, the plicae of cecum body were observed with short villi and the crypt glands became more developed (Fig.3). The lymphoid infiltrations of the lamina propria were observed in CBC at day 10 of post-hatch, but these only appeared at day 20 of post-hatch in the MVC.

Scanning Electron Microscopic Observations

The shape of the duodenal villi of day-old chicks showed finger-like projections in both breeds (see Fig.4). The side-appearance of villi surface showed transverse recesses in CBC (Fig.5A) and with a marked corrugated surface for MVC. The corrugated surface of the villi on higher magnification showed discontinuity and disruption of the epithelial mucosa (Fig.5B). In CBC, however, it was restricted to the tips of the villi, while the remainder villus surface showed clear penta or hexagonal outlines demarcation (Fig. 5C and Fig.5D). In higher magnification, the villi tips showed cluster of dome-shaped cells and cells protuberances around the central sulcus (Fig.6). At day 10 of post-hatch, the duodenal villi appeared to have leaf-like shape with a slightly curved narrow tip in CBC without appearance of recesses on the surface as seen clearly in MVC which still showed finger–shape villi (Fig.7). The side surface of villi in both breeds appeared rough with a clear cell outline. The tips of the leaf-like shape duodenal villi for CBC

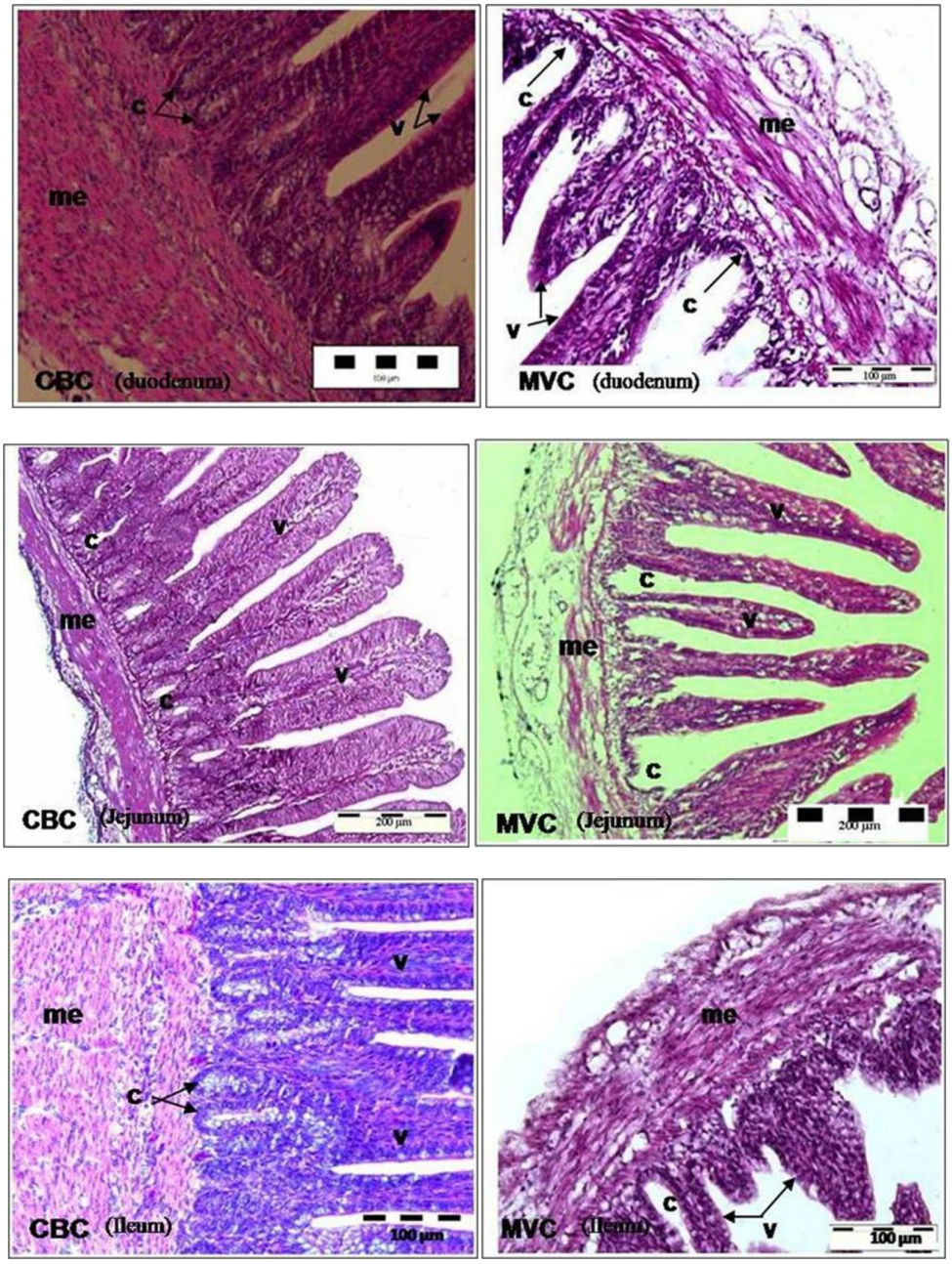

Fig.1: Microphotographs of the small intestine (Duodenum, Jejunum and Ileum) of day-old CBC and MVC shows the villi (v), the crypt (c) and muscularis externa (me) that are well developed in the CBC compared to MVC. H&E stain.

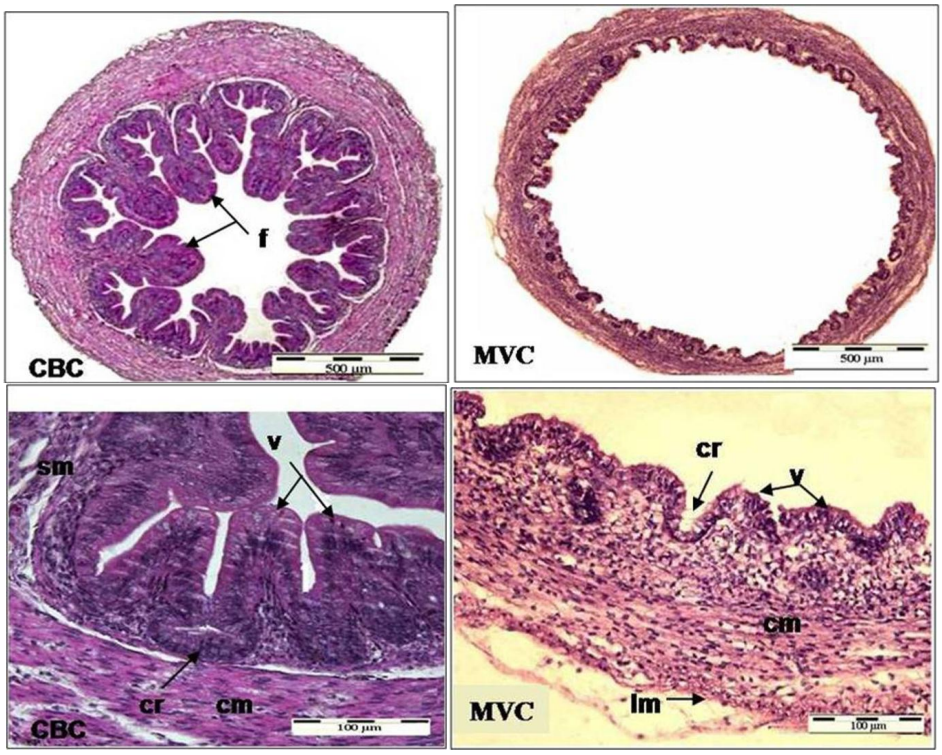

Fig.2: Microphotographs of the cross sections through cecal body of day-old CBC and MVC (upper) and similar sections with higher magnification (lower) shows the variation in hight of villus and fold, and the crypt depth between the breeds. f, fold; v, villi; cr, crypt; sm, submucosa; cm, circular muscle fibres; lm, longitudinal muscle fibre. H&E stain.

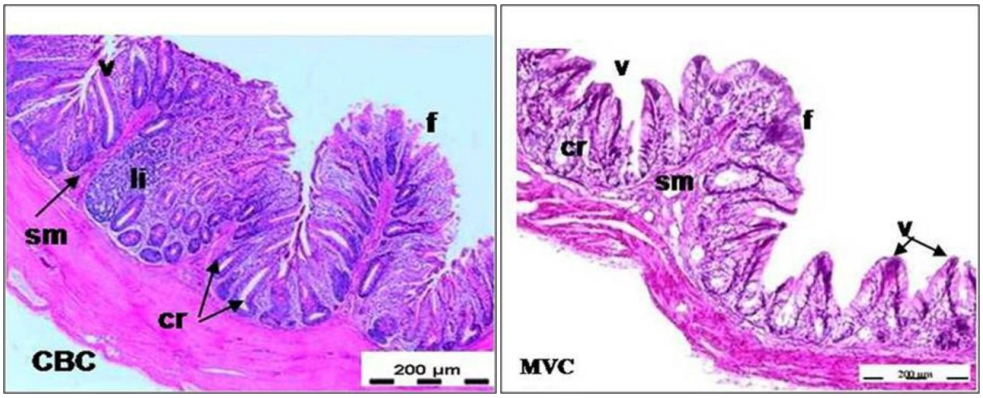

Fig.3: Microphotographs of the cecal body of 10 day-old CBC and MVC shows the long folds (f) and lymphoid infiltration (li); v, villi; sm, submucosa. H&E stain.

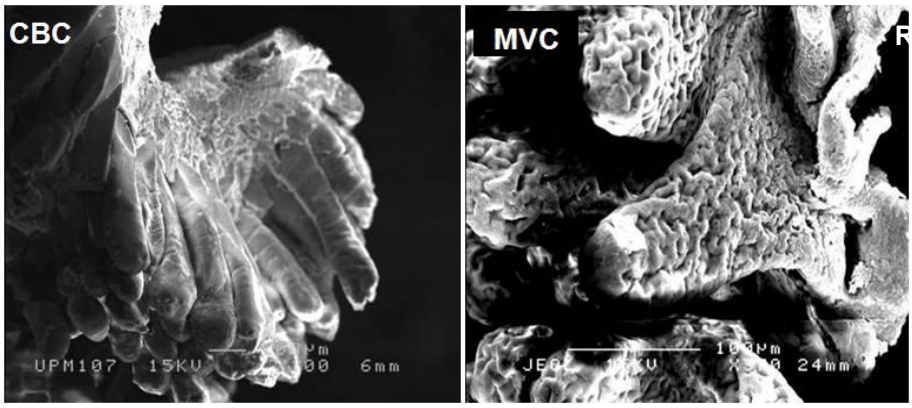

Fig.4: Scanning electron micrographs of the duodenal villi of day-old CBC and MVC shows finger-like villi, with transverse recesses in the CBC and with a marked corrugated appearance in the MVC.

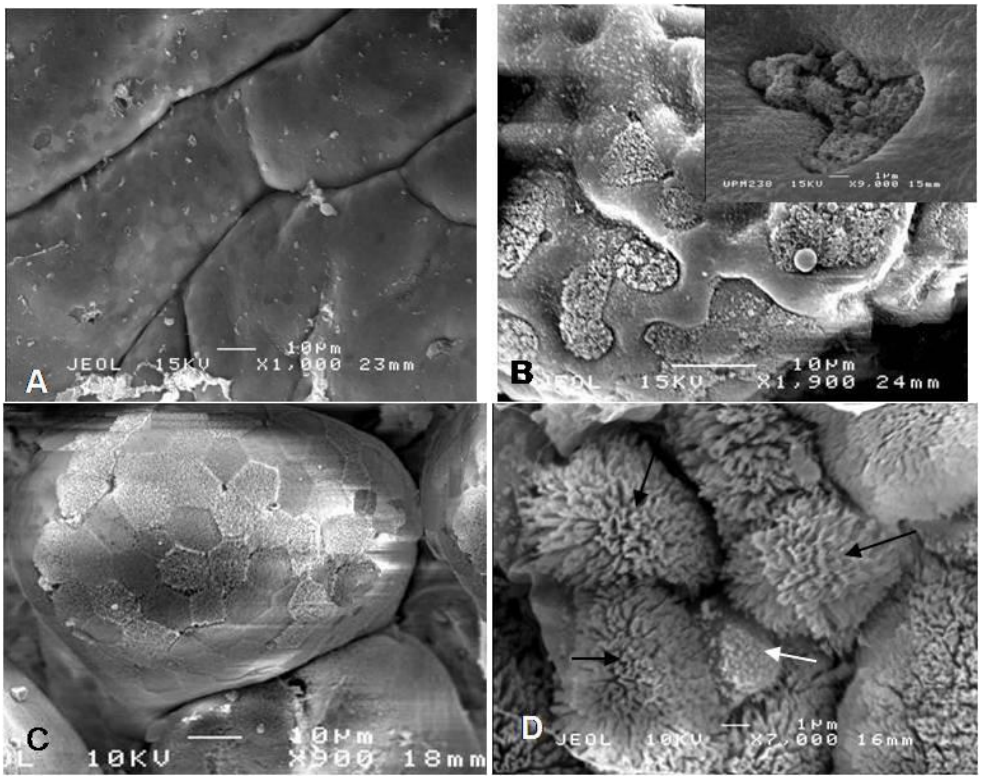

Fig.5: Scanning electron micrographs of the villi epithelium from different intestinal segments shows (A) villus surface with recesses, (B) discontinuities of the epithelial surface in the villus tip. Inset shows an enlarged view of an epithelial crevice (Bar 1 µm x 9.000), (C) villi tip with clear hexagonal or pentagonal cell outline, D) epithelial cells with long (black arrows) and short (white arrows) microvilli.

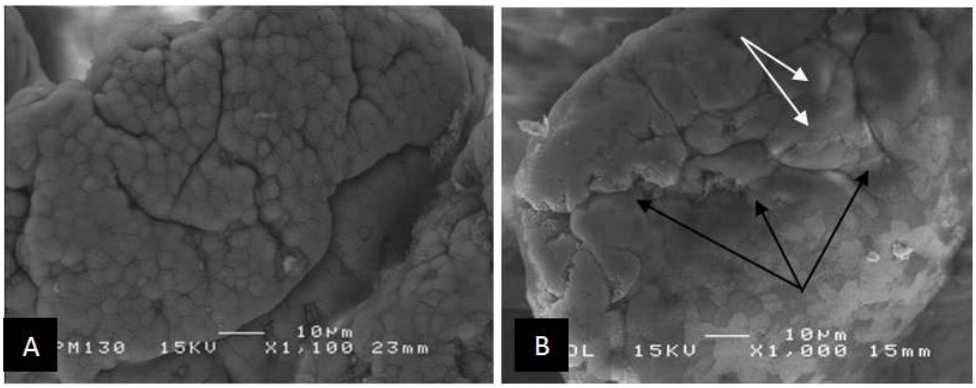

Fig.6: Scanning electron micrographs of the villi epithelium from different intestinal segments shows (A) cluster of dome-shaped cells in the villus tip, (B) rough villus tip, cells with protuberances (white arrows) around the central sulcus (black arrows).

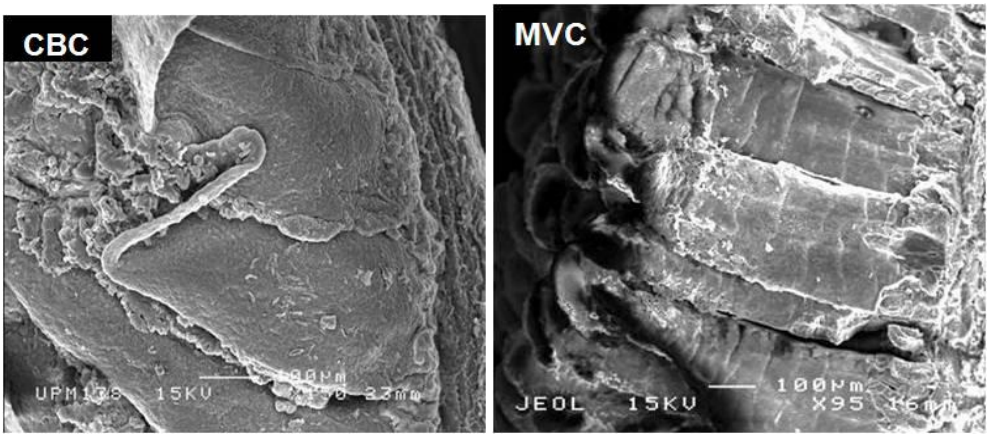

Fig.7: Scanning electron micrographs of the duodenal villi of 10 day-old CBC and MVC shows leaf-like in shape with a slightly curved narrow tip in the CBC, finger-like shape with a little compress on both sides, and appearance of the recesses on the surface in both the MVC.

were markedly curved above each other at day 20 of post-hatch (see Fig.8). The smooth surface replaced the recently exfoliated cells was commonly observed with deeper cells at these sites with short microvilli (Fig.9A). In MVC, however, the exfoliated zone (Fig.9B) was restricted on the tips of the tongue-like shape villi (Fig.8).

The jejunal villi of day-old and 10 day-old chicks of both breeds appeared similar to that of the duodenal villi of the same age. In 20 day-old chicks, the jejunal villi showed plate-like and tongue-like shape for CBC and MVC, respectively (Fig.10). However, the recesses and wide curled tips with cell activities represented by dome-shaped cells

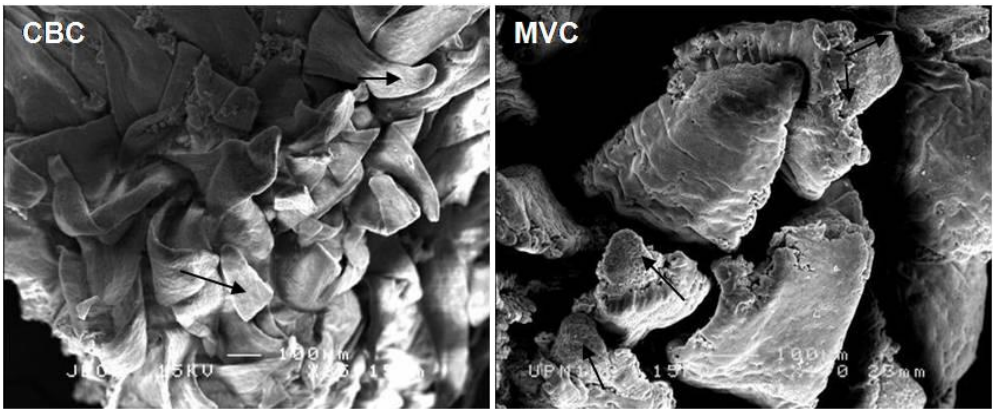

Fig.8: Scanning electron micrographs of the duodenal villi of 20 day-old CBC and MVC shows leaf-like shaped villi in CBC with curved tips above each other. Tongue-like villi with surface recesses and exfoliated area (arrows) in the MVC.

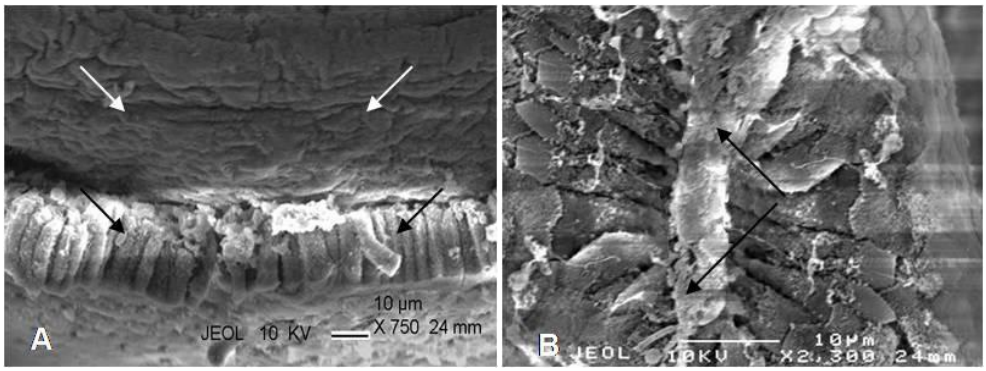

Fig.9: Scanning electron micrographs of the villi epithelium from different intestinal segments shows (A) exfoliated zone in the proximal part of villi showing the epithelial cells at this zone (black arrows) and the deeper cells (white arrows) which recently replaced the exfoliated cells, (B) exfoliated zone (arrows) restricted to the central area of the villus tip.

with cell protuberances were observed around the central sulcus particularly in MVC (Fig6).

The ileal villi of day-old chick of CBC appeared similar to the duodenal villi but without any surface recesses, while in MVC, a marked corrugated surface and areas of discontinuities were dominantly seen on the ileal villi (Fig.11). In 10-day-old chicks of MVC, the epithelial surface of finger-shaped ileal villi showed very clear crevices and discontinuity (Fig.12). The cell activities and exfoliated cells on the wide villi tips were also observed. However, these cell activities were commonly observed in the tongue-like villi of CBC at this age. In the 20-day-old chicks of CBC, the ileal villi appeared in a tongue-like shape with marked recesses and cells activities, while the villi of MVC still appeared to have finger-like shape (Fig.13).

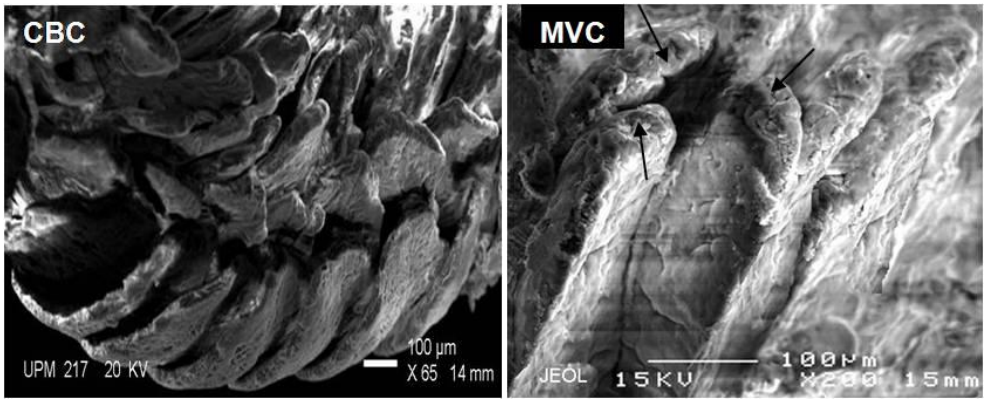

Fig.10: Scanning electron micrographs of the jejunal villi of the CBC and MVC at day 20 post-hatch shows the plate-like villi with wide tips in the CBC, tongue-like villi, thick with wide curled tips (arrows) in the MVC.

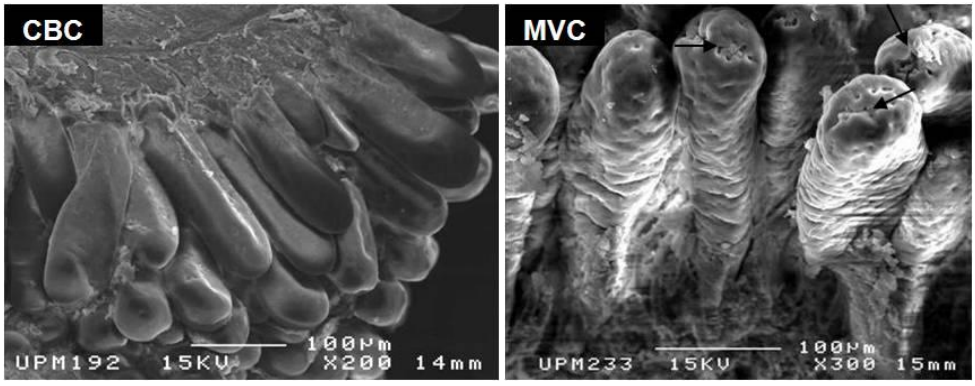

Fig.11: Scanning electron micrographs of the ileal villi of day-old CBC and MVC shows finger-like villi with smooth surface in the CBC, with a marked corrugated surface and areas of discontinuities (arrows) particularly on the villi tips in the MVC.

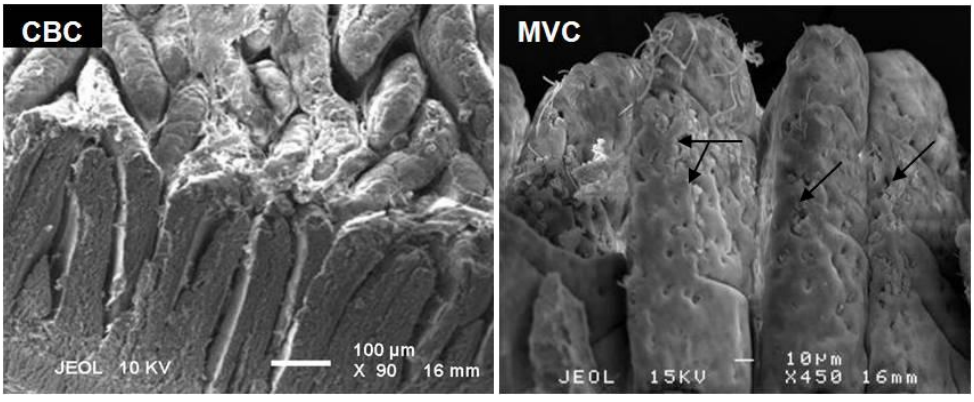

Fig.12: Scanning electron micrographs of the ileal villi of 10 day-old CBC and MVC shows the tongue-like villi in the CBC and finger to tongue-like villi in the MVC. Both breeds show curled villi tips, area of discontinuities on the epithelial surface (arrows) in the MVC.

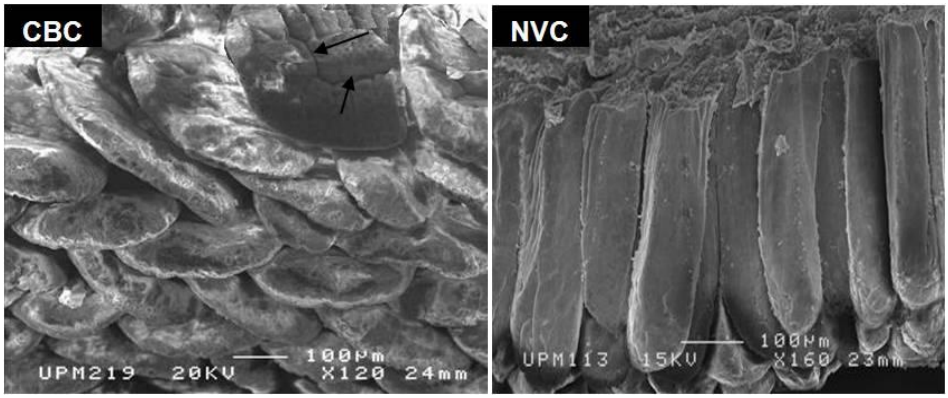

Fig.13: Scanning electron micrographs of the ileal villi of 20 day-old CBC and MVC shows tongue-like villi with marked recesses (arrows) in the CBC, finger-like with a little compress on two sides in the MVC.

DISCUSSION

In our experiment, the interior surface of the intestine of both breeds consisted of villi and lacked folding as seen in large mammals, and this observation is line with that of King and McLelland (1979) and Turk (1982). The results of the present study are also similar to the finding of Gussekloo (2006) who recognized four types of cells that constituted the epithelial mucosa. Through the histological slides, the intestinal villi showed more development in CBC than that of MVC. James *et al*. (1988) and smith *et al*. (1990) reported that in line selected for high growth rate, modification of villus structures occur toward increases in villus size and finally increases the intestinal absorptive surfaces. These in role are related directly to metabolic requirements for rapid body growth (Mayhew & Middleton, 1985).

From this study, the intestinal villi and crypts of day-old chi cks were less developed in MVC than in CBC, especially in the ileum. Similar result was also reported by Uni *et al*. (1999) in poult where the ileum show less development feature than other segments at first day of post-hatch. In rats and mice, crypts develop during the early postnatal period from the flat intervillus epithelium (Mathan *et al.*, 1976; Calvert & Potheir, 1990) but crypts in hatching chicks are not fully defined until 24 h post-hatch (Geyra *et al*., 2001). Smith *et al*. (1990) reported that between the selected breeds for high body weight and unselected one, there are differences in the crypt size of the small intestine, which are also reported in this experiment. Meanwhile, Yamauchi *et al.* (1996) reported that the ileum seems to be inactive in absorptive function in leghorn breed.

Our data are in line with those of Ogiolda *et al.* (1998) who reported that the thickness of the muscularis externa of the intestine is greater in heavy lines than in light lines. Karasova and Diamond (1983) and Watkins *et al*. (2004) explained that

broiler chickens and commercial ducks increases their nutrient transporter capacities by increasing the mass of the entire small intestine as a kind of adaptation to the large volume of food eaten. However, their tunica muscularis and serosa do not seem to be different from those of other birds (Chikilian & De Speroni, 1996; Gussekloo, 2006).

Our observations are similar to those of Rezaian and Hamedi (2007) who showed shorter villi and fewer glands found within the mucous membrane of the middle part of the cecum. In the present study, the cecum body showed a thin wall and poorly developed villi and crypt depth, particularly in MVC. The findings of Kitagawa *et al*. (1996) and Looper and Looper (2005) revealed that the distal two thirds of the cecum undergo regression and involve the atrophy of the epithelium and glands, while the villi never represent any typical appearance.

As for the SEM observation, the results of this study agree well with the findings of Yamauchi and Isshiki (1991) who reported that in a day-old chick, the intestinal villi showed finger-like projections in both heavy and light breeds of chicks. Bayer *et al*. (1975) revealed plate-like villi in day-old broiler chick. The villi, particularly in the duodenum, showed more matured surface epithelial cells in CBC than in MVC even at day 1 of post hatch. These observations seem similar to the findings of Yamauchi *et al.* (1992) and Yamauchi (2002), who showed that at day-old post hatch, the maturation of epithelial cells in light line is involved in the process of cell maturation but epithelial cells of villi in heavy line are almost matured at hatching. The histological alterations related well to the intestinal function at SEM level, represented by characteristic folds and numerous recesses (convoluted surface) (Bayer *et al*., 1975), however, the presence of rough surface, protuberated cells, dome-shaped cells, clear cell outlines, cell exfoliated, extrusion area, and cell clusters were frequently observed (Shamoto *et al*., 1999; Samanya & Yamauchi, 2001; Maneewan & Yamauchi, 2003). Furthermore, the results are also in agreement with the report by Yamauchi and Isshiki (1991) that the broiler breed showed more developed epithelial cell protrusions over the whole apical surface of the intestinal villi. In light breed, the protrusions were not so apparent and located only in the central area of the villus tip. The results of the present study, unlike CBC the villi of MVC at day-old chicks, showed marked corrugated appearance. Bayer *et al.* (1975) demonstrated that during early development stage, the villi show sub-epithelial crevices and discontinuity which is seen among some epithelial cells. The data of the present study, nonetheless, disagreed with the reports of Yamauchi and Isshiki (1991) that the intestinal villi is plate-like at day 10 of post hatch in heavy and light lines breeds. However, as in the finding of Yamauchi (2002) for day 10 broilers, the total proximal part of jejunal villi in the current work showed a complete replacement by deeper cells at the sites of exfoliated cells with unclear cell outline. Meanwhile, the villi of MVC showed cell

activities were observed around the central sulcus.

In our results, no much further development takes place in regards to villi shape at day 20 as compared to day 10 chicks for broiler breed. According to reports of Yamauchi and Isshiki (1991) in heavy and light lines, villi develop a plate-like shape in the duodenum, a wave-like shape in the jejunum and a tongue-like shape in the ileum for day 30 chicks. However, they stated that the fundamental villous shape and arrangement seem to be accomplished by day 10 of post hatch.

CONCLUSION

From the light and SEM observations, it was obvious that the intestinal mucosa of the CBC showed earlier development and more active villi than in MVC. Meanwhile, the intestinal mucosa of the newly hatched chick of the latter breed was in a process of development and maturation. The epithelial cells of the intestinal mucosa in CBC were more active for digestive and absorptive functions and they were also found to have reached morphological maturation earlier and faster than those in MVC, in which these alterations may have a great importance in the short life of the broiler breed associated with rapid growth.

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Morphological and Leaf Epidermal Features of Some *Capsicum* Species (Solanaceae) from Nigeria

Aworinde, D. O.[1]*, Ogundele, A.[2] and Ogundairo, B. O.[3]

[1]*Department of Biological Sciences, Ondo State University of Science and Technology, Okitipupa, Ondo State, Nigeria*
[2]*Department of Biology, Federal College of Education (Special), Oyo, Nigeria*
[3]*Department of Biological Sciences, Federal University of Agriculture Abeokuta, Ogun State, Nigeria*

ABSTRACT

Investigation on the leaf epidermal morphological features was carried out on five species of the genus *Capsicum* in the family Solanaceae collected from different regions in the Southwestern part of Nigeria in search of taxonomic and diagnostic characters that could be employed for their classification and identification. The species are *C. annuum* L., *C frutescens* L., *C. chinense* jacq., *C. baccatum* L., and *C. chacoense* Hunz. All the species possessed either anomocytic or anisocytic stomata type on both adaxial and abaxial surfaces except for *C. chinense* which showed paracytic stomata type on both surfaces. The leaves of *C. baccatum, C. annuum, C. chinense* and *C. frutescens* are amphistomatic, while that of *C. chacoense* are epistomatic. The similarities and overlaps observed in the cell shape, stomata index and anticlinal wall pattern of the species provide evidence for their genetic and evolutionary relationship and justification for their taxonomic grouping.

Keywords: Anatomy, *Capsicum*, Leaf epidermal, Macro-morphology, Micro-morphology, Nigeria, Solanaceae, Taxonomy

INTRODUCTION

The genus *Capsicum* belongs to the family Solanaceae. Members of the family are mostly herbs and shrubs and are usually cultivated as annuals (Dutta 2005). The larger genera are *Solanum* (1,500spp), *Cestrum* (250spp, mostly American), *Physalis* (100spp), *Nicotiana* (100spp) and *Capsicum* (50spp) (Dutta, 2005). The family is widely distributed throughout tropic and temperate region of the world with centre of diversity occurring in central in Central and South America and Australia (Aunay, 2001).

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E-mail addresses:
davidaworinde@yahoo.com (Aworinde, D. O.),
ogundele_ayomide@yahoo.com (Ogundele, A.),
odundairo@yahoo.com (Ogundairo, B. O.)
* Corresponding author

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Capsicum is an economically important genus in the Solanaceae, encompassing around 25 species, native to tropical and temperate regions of the Americas (Aunay, 2001). Five of its members *C. annum, C. frutescens, C. chinense*, *C. baccatum*, and *C. pubescens* were domesticated by American Indians and were exploited in global scale after Columbus owing to their valuable fruits, with pungent fruits being used as spices and sweet ones as vegetables, i.e. they are mostly cultivated worldwide because of their spicy and nutritional value. In addition, the genus has medicinal and ornamental uses. The stem is erect, woody and hairy; leaves are alternate, simple and exstipulate. The fruit is a berry and the seeds are minute endospermic with a straight or curved embryo. The taxonomic value of epidermal morphology is well documented in botanical literature (Mbagwu & Edeoga, 2006; Adedeji, 2004). The result of epidermal characters studied by Nwachukwu *et al*. (2007) in *C. annuum* and *C. frutescens* showed little variation except for their number of stomata. Adedeji *et al*. (2007) also reported that *C. annuum* can be separated from *C. frutescens* and *C. chinense* on the basis of the organographic distribution of their trichomes.

Information on detailed anatomical structures of the genus is still fragmentary; hence, this present work provides a useful means of generating data to enhance further identification and delimitation of the taxa in the region.

MATERIALS AND METHODS

The seeds used were collected from four different states in the southwestern Nigeria. They were sundried, sown and then raised to maturity on a small portion of land from where matured leaf samples were collected for the study. Collections were also made from the field to facilitate comparison.

Matured leaves were measured for each species at comparative position. Macro characters measured include leaf length and width (taken at a widest part of the leaf) and petiole. Qualitative characters such as leaf shape, margin and apex were also noted. Micro characters such as number of epidermal cell per view (x 400), thickness of the cell wall, size of the epidermal cell (at the widest point), number of stomata per view (x 400), length and width of stomata and stomata index were also measured.

Epidermal preparation was by obtaining sizeable portion (5mm²-1cm²) of the mature leaves from the transverse section of each leaf soaked in concentration solution of trioxonitrate (v) acid (HNO_3) for a period of about 18-24 hours. The appearance of air bubbles on the surface of the leaf fragments indicated their suitability for separation. They were then transferred into water in a Petri dish from where the upper and lower epidermises were carefully peeled. Selected specimens were stained in Safranin and counterstained in Fast green. Stained specimens cleared in clove oil to remove excess stain were mounted on slides with DPX mountant.

Stomata frequency was estimated from an average of ten counts. Length and

width of stomata were measured at X400 magnification using ocular micro-meter. The measurements were later converted to microns using a pre-calibrated stage micrometer.

The stomata index (I) was obtained by expressing the number of stomata per unit area as a percentage of the total number of cells in the same unit area using Salisbury (1927) method as modified by Hussin *et al.* (2000).

$$I = \frac{S}{S + E} \times 100$$

Where, I represents the stomata index, S, number of stomata per unit area and E, number of ordinary epidermal cells in the same unit area.

RESULTS

Macromorphological Characters

The leaves of the species examined are mostly simple and alternate with all possessing an entire margin. The leaf shape ranges from ovate to lanceolate. *C. annuum, C. chinense* and *C. frutescens* are pubescent, while *C. baccatum* and *C chacoense* possess glabrous leaf surface (Table 1). A thorough study of the species revealed variation in the sizes of leaves of each as the largest was observed in *C. chinense* and the smallest in *C. frutescens*. From the analysis carried out on the length, the largest is *C. annuum* (7.6 cm) and smallest in *C. chacoense* and *C. chinense* with both having a record of 2.4 cm. The lowest length/width ratio is 1:1 in *C. chacoense, C. annuum* with 2:1, *C. frutescens,* with 3:1 and the highest in *C. chinense* with 4:1 (Table 2). *C. Chinense* has the longest petiole and fruit stalk with the mean number of 13 cm and 11.4 cm repectively, while *C. chacoense* has the shortest petiole with the mean number of 6.1 cm, and *C. annuum* has the shortest fruit stalk of 4.3 cm (see Table 2).

Epidermal Cell

The shapes of epidermal cells on both the adaxial and abaxial surfaces are polygonal and mostly irregular in all the taxa. The anticlinal walls are curved, slightly straight and straight. The irregular cells usually have curved to slightly straight anticlinal

TABLE 1
Comparison of some qualitative morphological features of *Capsicum*

Taxa	Character codes			
	1	2	3	4
C. annuum	+	–	–	+
C. chinense	+	–	–	+
C. frutescens	+	–	–	+
C. baccatum	–	+	+	–
C. chacoense	–	+	+	–

Legend to character codes: 1 = Ovate leaf shape, 2 = Lanceolate leaf shape, 3 = Pubescent leaf, 4 = Glabrous leaf, + = Presence, − = Absence

TABLE 2
Morphological characters of the studied specimens [min (mean ± S.E) max]

Taxa	Leaf length (cm)	Leaf width (cm)	Petiole length (cm)	Fruit length (cm)	Fruit stalk (cm)
C. annuum	6.7(8.3 ± 0.3)10	3.1(3.3 ± 0.4)3.5	7.8(9.9 ± 0.4)11	2.4(2.9 ± 0.2)3.8	3.2(4.3 ± 0.3)5.7
C. chinense	6(8.9 ± 0.7)12	2.8(4.6 ± 0.5)6.5	9.2(13 ± 0.9)17	7(8.5 ± 0.2)9.4	9(10.5 ± 0.3)12.3
C. frutescens	5.4(5.9 ± 0.9)6.4	1.2(1.7 ± 0.8)2	6.9(7.6 ± 0.2)8.3	5.5(5 ± 0.1)6.4	7.8(8.3 ± 0.1)8.6
C. baccatum	5(5.7 ± 0.2)6.5	3(3.4 ± 0.1)3.9	6.6(7.2 ± 0.2)7.8	2.4(2.8 ± 10.1)3	4.4(5.1 ± 0.2)5.8
C. chacoense	4.5(5.3 ± 0.2)5.9	1.3(1.4 ± 0.1)1.6	5(6.1 ± 0.2)6.8	7.5(9 ± 0.3)11	10(11.4 ± 0.4)14

TABLE 3
Comparison of some epidermal features of *Capsicum* species (Adaxial)

Taxa	Character codes						
	1	2	3	4	5	6	7
C. annuum	+	-	+	-	+	-	-
C. chinense	+	-	-	-	-	+	-
C. frutescens	-	+	-	+	+	-	-
C. baccatum	+	-	-	+	+	-	-
C. chacoense	+	-	+	-	-	-	+

Legend to character codes: 1 = Anomocytic stomata, 2 = Anisocytic stomata, 3 = Irregular cell shape, 4 = Polygonal cell shape, 5 = Slightly straight anticlinal wall, 6 = Curved anticlinal wall, 7 = Curved/slightly straight anticlinal wall, + = Presence, − = Absence.

TABLE 4
Comparison of some epidermal features of *Capsicum* species (Abaxial)

Taxa	Character codes							
	1	2	3	4	5	6	7	8
C. annuum	+	-	-	+	-	-	-	+
C. chinense	-	-	+	+	-	+	-	-
C. frutescens	+	-	-	+	-	-	+	-
C. baccatum	-	+	-	+	-	-	-	+
C. chacoense	-	-	-	-	+	-	+	-

Legend to character codes: 1 = Anomocytic stomata, 2 = Anisocytic stomata, 3 = Paracytic stomata, 4 = Irregular cell shape, 5 = Polygonal cell shape, 6 = Slightly straight anticlinal wall, 7 = Curved anticlinal wall, 8 = Curved/slightly straight anticlinal wall, + = Presence, − = Absence.

TABLE 5
Variations in the epidermal cell sizes and cell wall thickness of the studied species

Taxa	Cell wall thickness per µm [min (max ± S.E) max]		Number of cell [min (max ± S.E) max]	
C. annuum	AD	AB	AD	AB
	2(3.3 ± 0.3)4	1(2.5 ± 0.3)4	48(56 ± 1.6)52	151(169 ± 4.4)189
C. chinense	1(2.6 ± 0.4)5	5(5.7 ± 0.3)7	8(14.8 ± 1.4)21	146(154 ± 1.5)160
C. frutescens	5(6.2 ± 0.4)8	1(3.6 ± 0.5)6	127(146 ± 3.6)160	88(97.3 ± 3.6)126
C. baccatum	3(4 ± 0.2)5	5(6.1 ± 0.3)7	46(62.7 ± 2.5)73	108(208 ± 5.5)235
C. chacoense	4(4.9 ± 0.3)6	1(2 ± 0.3)3	90(117.5 ± 5.3)136	15(19.4 ± 1.1)26

walls, while the polygonal cells usually have straight anticlinal walls. The thickness of the epidermal cell walls ranged from 2.6 µm in *C. baccatum* to 6.2 µm in *C. chacoense* on the adaxial surface and from 2.0 µm in *C. chinense* to 6.1 µm on the abaxial surface of *C. frutescens*. The number of cells on the abaxial surface is more than those on the abaxial surface of the studied species. The mean number ranged from 14.8 in *C. baccatum* to 146.6 in *C. chacoense* on the abaxial, while it ranged from 19.4 in *C. chinense* to 208.5 in *C. frutescens* on the adaxial (see Table 4).

Stomata

Fig.1 (a to j) show variation in the distribution and type of stomata among the taxa. Of the five species studied, *C. baccatum, C. annuum, C. frutescens* and *C. chinense* showed amphistomatic leaves, while only *C. chacoense* is epistomatic (Table 6). Most of the species possessed anomocytic stomata and anisocytic stomata type with only the abaxial surface of *C. chinense* possessing paracytic stomata.

DISCUSSION

The results of the morphological and epidermal features of the genus *Capsicum* studied revealed some stable diagnostic characteristics that could be used for taxonomic decision.

The variations in the type of trichomes possessed by the species is in accordance with the suggestion made by Roe (1971) that the genus exhibits great hair diversity which have been of considerable importance in

TABLE 6
Stomata characters for the studied taxa

Taxa	Stomata Length		Stomata Width		Stomata density		Stomata index %	
	AD	AB	AD	AB	AD	AB	AD	AB
C. annuum	42(48.7 ± 1.27)55	65(73.5 ± 1.68)82	20(25.9 ± 0.84)30	21(24.4 ± 0.67)27	10(13.8 ± 0.85)18	37(42.3 ± 1.05)48	19.8	20.0
C. baccatum	58(61.2 ± 0.83)65	52(61.1 ± 1.57)68	20(22.2 ± 0.55)25	20(24.2 ± 0.73)28	3(4 ± 0.26)5	21(33.6 ± 2.41)46	21.3	17.9
C. chacoense	65(74.9 ± 2.03)85	–	30(36.6 ± 1.20)41	–	8(11.8 ± 1.06)17	–	7.5	–
C. frutescens	50(60.5 ± 1.46)66	58(68 ± 2.41)80	18(22.2 ± 0.76)25	25(29.4 ± 1.21)35	11(15.3 ± 0.87)19	48(52.9 ± 1.08)58	19.6	20.2
C. chinense	50(58.3 ± 1.45)62	70(76.7 ± 1.63)85	20(24.3 ± 0.91)28	10(10.8 ± 0.29)12	25(30.8 ± 1.02)35	3(4.3 ± 0.34)6	20.8	18.1

- denotes no stomata present

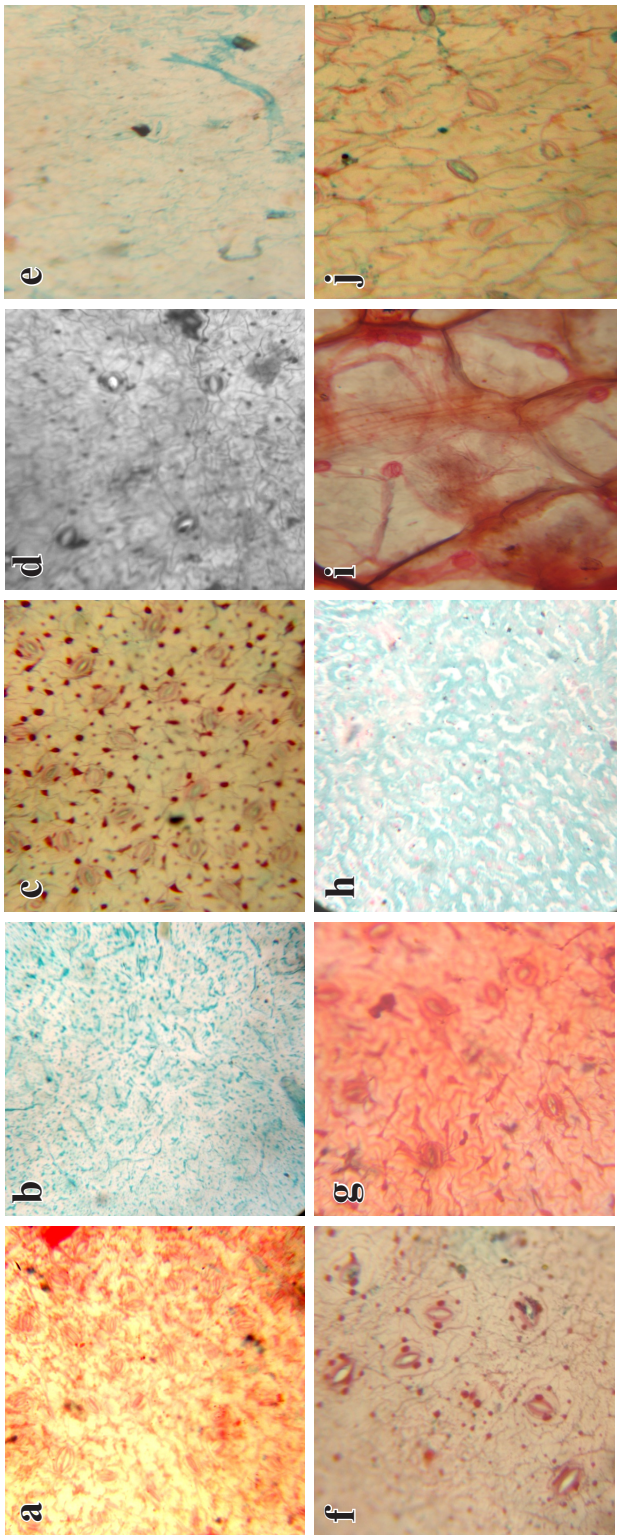

Fig.1 a) Abaxial surface of *C. annuum* showing Anomocytic stomata and irregular cell.
b) Adaxial surface of *C. annuum* showing Anomocytic stomata and irregular cell
c) Abaxial surface of *C. frutescens* showing Anomocytic stomata and irregular cell
d) Adaxial surface of *C. frutescens* showing Anisocytic stomata and polygonal cell
e) Abaxial surface of *C. chinense* showing paracytic stomata and irregular cell
f) Adaxial surface of *C. chinense* showing Anomocytic stomata and irregular cell
g) Adaxial surface of *C. chacoense* showing Anomocytic stomata and irregular cell
h) Abaxial surface of *C. chacoense* showing no stomata and polygonal cell
i) Adaxial surface of *C. baccatum* showing Anomocytic stomata and polygonal cell
j) Abaxial surface of *C. baccatum* showing Anisocytic stomata and irregular cell

comparative investigations in angiosperms. They are frequently present, easily observable and have been found to have variation patterns which correlate with other features of the taxa under investigation.

Morphologically, the vegetative features that separate the species from one another is in line with earlier works of Okwulehi and Okoli (1999), and Edeoga and Emeka (2000), who used comparative morphology of different species in establishing relationships relation among various taxa. The cell wall and shape varied among the species; *C. chinense* and *C. annuum* can be grouped together as they possess irregular shape both on the adaxial and abaxial surfaces, while *C. baccatum* and *C. frutescens* can also be grouped by their polygoanal and irregular shapes on the adaxial and abaxial surfaces, respectively. *C. chacoense* is the only species that possess irregular cell shape on the adaxial surface and polygonal cells on the abaxial surface. Stace (1965) suggested that environmental conditions, such as humidity, play a significant role in determining the pattern of anticlinal walls. The anomocytic type of stomata present in the species of *Capsicum* indicates that the species are phylogenetically related (Nwachukwu *et al*., 2007). The preponderance of stomata on the adaxial surfaces is probably an adaptation to water loss (Mbagwu *et al*., 2008). This is in agreement with Metcalfe and Chalk (1979), as well as Mbagwu and Edeoga (2006), who observed that stomata are usually more on the lower epidermis in the species of *Amaranthus* and *Vigna,* respectively.

Variation was also noted in the stomata density and index among species which is often a reflection of physiological responses, together with the combination of environmental factors and this can be of help in delimitation at the species level (Adegbite, 2008). Metcalfe and chalk (1979) suggested that while stomata density varies considerably with the age of leaf, stomata index is highly constant for a given species.

Variation shown by the data and figures of the characters such as stomata density, stomata index, stomata type, stomata length and width are therefore of taxonomic importance. Also, the similarities observed in stomata type and shape provide evidence for their genetic and evolutionary relationships and justification for their taxonomic grouping.

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Human-Macaque Conflict at the Main Campus of Universiti Kebangsaan Malaysia

Md-Zain, B. M.*, Ruslin, F. and Idris, W. M. R.

School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia

ABSTRACT

Long-tailed macaques and students at the Universiti Kebangsaan Malaysia (UKM) (National University of Malaysia) main campus have lived together for the past forty years. Overlapping niches between the two sides have caused conflicts between the university residents and long-tailed macaques. In this study, questionnaire surveys were distributed to eight student residential colleges, involving about 776 students. Awareness, perceptions, opinions, and secondary observations of the nuisance behaviour of long-tailed macaques, and the effectiveness of precautionary measures were discussed, based on this survey. The results indicate that three-quarters of the respondents are fearful of the macaques. Less than 10% favoured the macaques' presence on campus, and 15% of the respondents supported the eradication of the population. Half of the respondents suggested that precautionary measures, such as improving waste management and macaque translocation, would be effective methods for managing their nuisance behaviour.

Keywords: Long-tailed macaques, *Macaca fascicularis*, nuisance, pest, student perception

INTRODUCTION

Long-tailed macaques (*Macaca fascicularis*) are known to dwell near the fringes of forest patches and are popularly perceived as pests (Md-Zain *et al*., 2011). These macaques disturb local people by exploiting unprotected human facilities such as waste dumps and entering unoccupied houses (Md-Zain *et al*., 2011) and harassing humans with food cues, such as biting and scratching, as well as stealing food (Sha *et al*., 2009). These altercations could potentially result in zoonotic disease transmission (Engel & Jones-Engel, 2011), negative attitudes towards animal welfare and conservation (Webber *et al*., 2007)

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E-mail addresses:
abgbadd1966@yahoo.com (Md-Zain, B. M.),
fahanih20@yahoo.com (Ruslin, F.),
wmrazi@gmail.com (Idris, W. M. R.)
* Corresponding author

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and the damage of macaque-accessible properties (Malaivijitnond *et al*., 2011).

Previous human-macaque conflict studies have focused on the relationships between habituated macaques and the local community, explaining the variations in human traditions and capabilities in dealing with macaques and the counter-actions taken (Malaivijitnond *et al*., 2011; Sussman *et al*., 2011). Rhesus monkeys (*M. mulatta*) of the Aligarh Muslim University Campus (AMU) in India were reportedly involved in nuisance behaviour such as stealing clothes and edible items, and uprooting vegetables and gardens, with more prevalence in female hostels (Imam *et al*., 2002). The same situation occurred in the Gauhati University Campus in Assam, India, compounded by the problems of overpopulation, improper waste disposal and the easy availability of food, seeming to worsen the scenario, with increased incidents of biting (Devi & Saikia, 2008). Long-tailed macaques at the UKM main campus are semi-habituated (Md-Zain *et al*., 2010). Md-Zain *et al.* (2011) have recorded previous human-macaque incidences, including the latter foraging at waste dumps, entering buildings and displaying aggression toward humans. The macaques often foraged in garbage cans, invaded rooms, and harassed local people or students, but no physical attacks, such as biting, were observed during the study by Md-Zain *et al.* (2011). Some mitigation measures were put into place, including the installation of wire mesh on the windows, instructions to avoid feeding the macaques, securing waste dumps and bin lids, and the early collection of trash.

Communities that are better prepared and have adequate precautionary measures against macaques are able to be proactive, when compared to the majority, who depend solely on the immediate action of DWNP. In addition to the previously implemented means of monitoring and controlling macaques that are available, local peoples' perceptions and experiences with the animals may provide a better assessment of observed counter-action and precautionary measures. Gathering information on the degree of human disturbance and attitudes of the local community may contribute to the formulation of appropriate measures of deterring macaques. The aim of this study is to assess the awareness, experience, attitudes, and perceptions of UKM students towards the macaques. Their attitudes and perceptions could determine the extent of damage that can be caused by the primates and these factors were measured and assessed in order to suggest decision-making options for the effective management of macaque nuisance behaviour on campus.

MATERIALS AND METHODS

Study Sites

UKM is located in Bangi, Selangor in Malaysia, and covers approximately 1096 ha. It is located near a lowland secondary dipterocarp forest known as the UKM Permanent Forest Reserve, with a size of 100 ha, surrounded by a golf course, an oil-palm plantation, and many small forest fragments among the clustered student residential colleges (RC): Burhanuddin Helmi, Ibrahim Yaakob, Aminuddin Baki, Rahim Kajai,

Ibu Zain, Keris Mas, Pendeta Za'ba, and Ungku Omar (Fig.1). The structured areas on the campus include the staff housing facilities (residential house of Bukit Puteri, PBP), administration buildings, faculties, clubhouse, health centre, visitor house, mosque, and two stadiums. Fig.1 shows that the UKM main campus has many forest patches, which host several other primate species such as pigtail macaques (*Macaca nemestrina*), banded leaf monkeys (*Presbytis siamensis*), and spectacled dusky leaf monkeys (*Trachypithecus obscurus*), as well as other animals (Md-Zain & Ch'ng, 2011).

Survey Questionnaire

The survey questionnaire included questions about student opinions, awareness, attitudes, and experiences with macaques in the residential colleges on the UKM main campus in June of 2010. Seven hundred and sixty-six people from eight RCs participated in the survey. There were no interviews conducted and the questionnaires were distributed randomly. Each respondent had stayed in the RC for at least one semester and only fully answered forms were accepted (766 respondents) and counted for the analysis. After the questionnaires were collected, the data was categorized and analyzed using Minitab® Statistical

Fig.1: Location of study sites indicating the appearance sites of *M. fascicularis*

1: Burhanuddin Helmi Residential College (BHRC), 2: Ibrahim Yaakob Residential College (IYRC),
3: Aminuddin Baki Residential College (ABRC), 4: Rahim Kajai Residential College (RKRC),
5: Ibu Zain Residential College (IZRC), 6: Keris Mas Residential College (KMRC),
7: Ungku Omar Residential College (UORC), 8: Pendeta Zaa'ba Residental College (PZRC).

Software (Minitab 14), and χ^2 analyses were used to test for differences in frequencies between the RCs. The number of the respondents was not consistent between the RCs, which ranged between 86 and 103 respondents each. The differences in the number of respondents were not large, and as such, it was not necessary to standardize the numbers. The proportions and percentages were presented, since all data was nominal.

RESULTS

The distributed surveys involved 78.4% female respondents and 22.6% males, ranging from 19 to 24 years of age. Ethnically, 77.2% are Malay, 14.8% Chinese, 4.6% Indian, and 3.4% of the respondents answered that they were of other races. Of the participants, 79.4% resided in the dorms, and the rest resided in apartments.

Sixty-one point six percent of the respondents (N = 776) could differentiate between long-tailed macaques and pig-tailed macaques, based on the written description provided in the questionnaire. Ninety-two point one percent of the students agreed that the most sighted raiders were sub-adults and adults. Sixty-one point six percent of the respondents could not differentiate between the sexes of the macaques, although there were significant differences at each study site (χ^2 = 27.479; df = 7; P = 0.000). The frequency of detection differed among the residents, depending on their ability to detect the presence of macaques (χ^2 = 306.534; df = 21; P = 0.000). Macaque foraging times, as monitored by the students near the colleges, were not uniform between the study sites (χ^2 = 97.442; df = 21; P = 0.000). The estimate of the number of macaques by the residents during foraging also differed between the RCs (χ^2 = 225.05; df = 21; P = 0.000). Ninety-seven point six percent of the respondents agreed that the macaques were present near the RCs in order to forage. Ninety-four point six percent of the respondents agreed that leftover food encouraged the foraging behaviour.

Harassment by macaques also differed between study sites; for some, more than 53% of the students were disturbed by the macaques (χ^2 = 51.536; df = 7; P = 0.000). The macaques were seen using a variety of ways to enter student rooms, and these methods were not uniform throughout RCs (χ^2 = 306.534; df = 21; P = 0.000); only 28% of the respondents noticed the macaques' presence. Fifty-seven point six percent of the respondents had experienced macaques entering their rooms, and 70.9% of them found their room untidy and cluttered, whereas 1.8% discovered faecal matter. Fifty-six percent reported items stolen by the macaques, and 93% of the residents disliked the presence of macaques near their hostels (χ^2 = 14.622; df = 7; P = 0.041).

Fifteen percent of the respondents had fed macaques before, although the percentage was different according to the site, with the highest percentages noted in the Ibu Zain RC with 30.1% (N = 31) and Burhanuddin Helmi RC with 25.6% (N = 22) (χ^2 = 45.821; df = 7; P = 0.000). About 91.4% of the respondents thought that the nuisance behaviour could affect both the

health and safety of the residents. About 94% of them agreed that the macaques could obtain a high quality diet in human areas as compared to forest patches, although this answer varied according to the site (χ^2 = 25.875; df = 7; P = 0.001). Fourteen point four percent of the respondents had complained to the office or RC administrator, while 80.2% (N = 622) had not made any complaints. Eighty-three point two percent acknowledged that the responsible parties, both the RC administrator and UKM security, were concerned about the situation (χ^2 = 16.145; df = 7; P = 0.024). Seventy-three point seven percent of the students agreed that primate translocation could prevent nuisance behaviour (χ^2 = 14.260; df = 7; P = 0.047); however, only 8.6% of the respondents agreed that culling could be effective in controlling the disturbances.

DISCUSSION

Based on the respondents' experiences, incidences with macaques most often involved one to ten macaques at a time (41.71%) (Table 1). This was followed by encounters with between ten and twenty macaques at a time (23.98%) (188 respondents). Approximately 34.31% of the respondents came across more than 20 macaques at one time. More than 98% of the students often saw a group of macaques in the evening. From these results, the researcher could infer that students had a higher probability of encountering more than 10 macaques at a time. This suggests that macaques prefer foragING in human areas in small subgroups, with frequent spreading of groups, since macaque group sizes in the UKM range from 18 to 56 individuals (Md-Zain *et al*., 2011).

This survey revealed that 28.6% of THE respondents could only confirm the presence of macaques when they came into sight. Twenty-three point five percent of the respondents, who were familiar with the macaques' nuisance behaviour, could predict the presence of macaques based on messy surroundings. Nineteen point two percent of the respondents could detect the presence of a macaque through the macaques' calls, and were able to warn others, which reduced losses and damage. Ninety-three point six percent of the respondents agreed that long-tailed macaques made the area dirty, and 91.4% agreed that their safety and health were at risk as a result of the macaques' nuisance behaviour.

Macaques often take waste bags and drag them away from the dumpsites, leaving them in corridors and drains, resulting in messy and smelly surroundings (Mastura, 2008). Eighty-six point nine percent of the students did not like the macaques because of their habit of tearing apart waste dumps and leaving areas dirty (Table 1). A study by Sha *et al.* (2009), in the Bukit Timah Nature Reserve in Singapore, found that about 13.4% of the total behaviour comprised of searching of bins, cars, and houses. The raiding of caged or protected garbage bins is irregular in Singapore based on observations by Schillaci *et al.* (2007). Closed but unsecured garbage bin lids did not help to deter macaque movements as they have a sharp sense of smell that enables them to detect the scent of food from far away.

About 60% of the students said that the situation had worsened over the current year, compared to the year before. On the other hand, 40% said that the macaques were tolerable for the moment. Respondents from the Rahim Kajai RC only saw the macaques near the bus stop and stated that they rarely visited the dormitory (Table 1). This raiding frequency could be influenced by the availability of food (Table 1). About 97.6% of the respondents believed that the macaques went to the RCs to look for food and this pattern has been recorded since 2003 (Table 1). Macaques leave the forest patches near the RCs, where they sleep, to forage the RC waste dumps. More than 90.4% of the respondents agreed that the possibility of the macaques visiting the residential colleges was the result of unplanned development and the presence of available food from waste bins (Table 1).

Approximately 41.7% of the students witnessed macaques opportunistically entering rooms when windows were left open, and 5.8% of these break-ins were due to damaged windows, while 15.3% of the break-ins occurred when the macaques were able to open the window. Thirty-seven percent of the students did not experience macaque break-ins. In the order of frequency, most students discovered messy rooms (39%), others found no mess, and 14 students found their rooms with macaque droppings and urine after a break-in. The survey responses indicated that students seemed to have gotten used to the after-effects of a macaque break-in.

The behavioural study showed that the frequency of aggression varied between colleges (Md-Zain *et al.,* 2011). More than 71 respondents from each RC felt frightened with the presence of macaques. One form of aggression shown was the chasing of students, which can sometimes involve the biting of those who are slow to run away. There are two primary reasons why the macaques chased the respondents. One was to steal food that the respondents were carrying (Sha *et al.,* 2009), and the other was to scare or force the respondents to leave as they were too near or encroaching the macaque group's perceived territory. Both situations scared the respondents, especially the females, and in one case a respondent who was trying to outrun a chasing macaque fell over. The results also showed that this aggression is the very reason why there is a fear of the species, as 76.2% of the respondents felt scared when the macaques were present, 53.3% of the students had been disturbed or chased by the macaques, and more than 80.15% of the respondents had seen people being chased.

Male macaques, particularly adults, pose a larger threat, as they defend their foraging group. This aggression is not welcomed by students, and any threatening behaviour towards the macaques may be perceived as provocation and lead to the macaques chasing and biting the students in return. About 18 (2.34%) of the respondents had been bitten by macaques, with an average of 2 students per residential college, and a deviation of about 1 or 2 students (Table

1). Recorded direct aggression towards the students was high, and this macaque-human interface may, in turn, promote primate to human disease transmission (Engel & Jones-Engel, 2011). In a random survey in Gauhati University Jalukbari, Guwahati, Kamprum, Assam, India, Devi, and Saika (2008) reported a total of 27 cases of monkey bite incidences and 49 cases of aggressive threats, with physical attacks in the form of scratching, biting and mass chasing of people. Forty-eight bites occurred during 420 provisions of food interactions with tourists in Padangtegal, Bali (Fuentes & Gamerl, 2005). There were 39 (2.1%) human-macaque interactions recorded to have resulted in bites in Gibraltar (Fuentes, 2006). In Singapore, Sha *et al*. (2009) reported that about 19.1% of macaque aggressive interactions included threats, chasing, and lunging. Two-thirds of these interactions were observed when a human was carrying food or indicated other food cues, and one-quarter occurred when a human provoked a macaque (Sha *et al.,* 2009).

The harassment of macaques by students who had been chased or threatened by adult macaques mostly took place in core areas when macaques roamed student passages to faculty buildings (Mastura, 2008; Idris, 2009; Ruslin, 2010). The macaque groups in Keris Mas RC and Aminuddin Baki RC were found to be less of a nuisance compared to other groups because the waste dumps are far from the students' paths and blocks. Places to perch are also not easily available to the macaques, making them less conspicuous to the students and reducing other potential dangers (Idris, 2009; Ruslin, 2010). In the evening, the macaques occupy forest patches near the bus stops and the pedestrian walks, and the students detected the presence of a troop of macaques from their sounds and visibility. Leaping and a quadruple limb posture displayed by adult alpha males were the most frequent warnings given to respondents (Thierry, 1985) when they failed to notice that they were approaching macaque foraging sites (Ruslin, 2010). Many respondents also stated differences in macaque group sizes as macaques usually separate into subgroups during foraging (Idris, 2009). Ruslin (2010) states that an observer can infer that female adults and juveniles prefer to be vigilant and on the lookout as they forage.

The macaques were rarely given food directly by humans, but they did have direct interactions with the students and did not engage or approach humans for food. Instead they preferred rummaging through garbage bins and felt fear towards humans, staying away even when they noticed students carrying food. The opposite occurs in tourist lodges, camps, temples, and monkey sighting sites, such as those in India, Bali, Gibraltar, and the Bukit Timah Nature Reserve in Singapore (Lee & Priston, 2005). These studies suggest that controlled behaviour and less provocation by humans towards macaques could result in commensal interactions between the students and macaques in the UKM.

Fifteen percent of the students had fed the macaques at least once (Table 1),

TABLE 1
Questions used in the survey distributed to the students from eight residential colleges on the UKM main campus.

Questions (N = 776)	Yes (%)	No (%)	SD		χ^2		
			Yes	No	Value	Df	P
Understanding and knowledge of students about macaques							
Can differentiate *M. fascicularis* and *M. nemestrina*	61.6	38.4	4.75	5.42	7.201	7	0.408
Can differentiate male and female macaques	38.4	61.6	9.06	9.6	27.479	7	0.000*
Estimated number of macaques when foraging							
1-10	41.5		24		225.050	21	0.000*
10-20	24.1		10.43				
20-30	12.9		7.67				
> 40	21.5		13.7				
State category of raiders often seen							
Adult	30.5		4.31		8.369	14	0.869
Sub-adult	61.6		6.30				
Juvenile	7.9		1.41				
State time for foraging near college							
Morning	7.7		3.63		97.442	21	0.000*
Afternoon	5.8		4.75				
Evening	58.5		13.63				
All of the above	28		11.65				
State ways to detect the presence of macaques							
Distinct sound	19.2		8.33		51.287	28	0.000*
Screen or window closed abruptly	13.7		8.55				
Macaques were visible	28.6		11.20				
Messy garbage	23.5		11.07				
No answer	15		28.4				

TABLE 1 (continue)

Questions (N = 776)	Yes (%)	No (%)	SD		χ^2		
			Yes	No	Value	Df	P
Students attitude concerning macaques							
Favour their presence	6.9	93.1	3.48	5.42	14.622	7	0.041*
More serious than last year	59.7	40.3	14.02	11.43	50.257	7	0.000*
Scared when macaques present	76.2	23.8	7.72	7.92	25.262	7	0.001*
Have feed macaques before	15	85	8.65	9.08	45.821	7	0.000*
Authorities are concerned about the situation	83.2	16.8	7.25	5.78	16.145	7	0.024*
Authorities have managed the situation responsibly	91.2	8.77	6.26	2.45	5.733	7	0.571
Have experienced nuisance behavior							
Leave the surrounding hostel messy	93.6	6.39	5.62	2.85	9.348	7	0.229
Macaques take property	56	44	3.18	28.5	Not valid for χ^2		
Harassed or chased	53.3	46.7	14.3	15.6	63.567	7	0.000*
Bitten by macaque	2.3	97.7	1.49	6.39	Not valid for χ^2		
Seen someone being disturbed by macaque	80.2	19.8	10.19	10.31	51.536	7	0.000*
Breaking-in, ways macaques seen entering a room							
Through open window	41.7		20.12		306.534	21	0.000*
Through damaged window	5.8		3.29				
Macaque opens window	15.3		11.57				
No answer	37.1		29.1				
Conditions of room after breaking-in							
No clutter	16.8		9.85		Not valid for χ^2		
Cluttered and untidy	39		18.28				
Leave stool	1.8		1.83				

TABLE 1 (continue)

Questions (N = 776)	Yes (%)	No (%)	SD		χ^2		
			Yes	No	Value	Df	P
Student responses in understanding nuisance behaviour							
Macaques were present for foraging	97.6	2.43	5.19	1.48	7.393	7	0.389
Nuisance behaviours could affect health and safety	91.4	8.6	6.06	1.92	4.689	7	0.698
Rubbish bins without proper lids encourage foraging	86.9	13.1	4.85	4.57	12.681	7	0.080
Food left over encourages foraging	94.6	5.42	5.08	2.63	10.526	7	0.161
High quality of diet in human areas, compared to forest patches	90.4	9.6	6.32	5.44	25.875	7	0.001*
Suggestions and favourable decisions to overcome problems							
Have made complaint	20	80	12.47	10.55	Not valid for χ^2		
Capture and transfer could prevent nuisance behaviour	73.7	26.3	7.23	6.39	14.260	7	0.047*
Poison and killing could be effective	8.6	91.4	6.59	9.13	40.085	7	0.000*
Proper steps to overcome the problems							
Putting up wire nets	23.7		9.95		197.788	42	0.000*
Reinforcement from DWNP	23.2		9.84				
Effective waste management	22.4		8.97				
Provide inaccessible waste dumps	17.4		7.02				
Cut down or trim trees	2.9		2.264				

which might have increased the occurrence of food-grabbing by the macaques. About 34.57% of the students stated that the macaques had taken things from them. The items stolen were mostly food, followed by beverages (cans, cartons, cups, and in plastic bags), books, notes, etc., as these were the items most often carried by the students and attracted the macaques' attention, possibly being mistaken for food. Observations by Chauhan and Pirta (2010) indicated that rhesus monkeys in urban areas were engaged in snatching and stealing non-edible objects from people as a strategy to obtain food in exchange. Monkeys were often observed taking away valuable things such as spectacles, mobile phones, purses, or shoes from passersby, and dropped them only when severely threatened or, more often, when given something edible in exchange (Chauhan & Pirta, 2010). These observations indicate that the tactics of rhesus monkeys are associated with the exchange of commodities (Drapier *et al.,* 2005; Chauhan & Pirta, 2010). In UKM, 86.47% of the stealing by the macaques involved food and beverages, while the rest involved the taking of books and notes. The surveys revealed differences between the residential colleges, with Aminuddin Baki RC and Pendeta Za'ba RC indicating that more than 70% of the students had not had items taken from them. In Aminuddin Baki RC and Ungku Omar RC, however, students had lost clothes from clothes lines and toiletries to the macaques.

The effectiveness of the RC administrators and UKM security in implementing control measures was questioned to clarify the students' perceptions of the party responsible for macaque nuisance controls. About 15.6% of the respondents complained to the residential college office and campus wardens. Another 4% complained to the student council and of UKM and DWNP (Table 1). Suggestions by the respondents to reduce the occurrences of nuisance behaviour varied between preventive, control, and intrusive measures such as installing wire nets on the windows. This kind of prevention is similar to the preventative measures taken by the residents in Vrindavan, India, who covered windows and open places with screens, barbed wire or iron grills to prevent rhesus monkeys from entering (Southwick *et al.,* 2005). This measure was preferred by the students (25.7%) as it is the most effective for all of the residential colleges. Twenty-eight percent of the students agreed that securing the bins could help to dissuade the macaques from rummaging, since the absence of food patches would discourage their presence in RCs. Meanwhile, culling by DWNP or scare tactics were suggested as reliable methods by 23% of the respondents in order to reduce disturbances. More than 76.3% of the respondents also believed that capture and translocation methods should help to control nuisance behaviours although the rest did not think that this was necessary (Table 1). About 8.6% agreed with the use of poison or culling to overcome the problem. About 3% of the respondents also suggested cutting down trees near RCs, since the macaques tend to take cover in the nearest tree while

foraging; however, the RC administrator has no plans to execute this suggestion. The students are partially affected by the nuisance behaviours but only 8.6% of them agreed that poison or culling could be effective in controlling the situation. The implementation of precautionary measures and effective waste management is crucial for the efficient long-term management of this pest species in UKM.

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The Amphibian Checklist of Bukit Larut, Perak, Malaysia

Shahriza Shahrudin[1]* and Ibrahim Jaafar[2]

[1]*School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia*
[2]*School of Distance Education, Universiti Sains Malaysia, 11800 Pulau Pinang, Malaysia*

ABSTRACT

One of the biodiversity hotspot areas in Peninsular Malaysia is Bukit Larut, which is located within the Banjaran Bintang (Range) near Taiping, Perak. The amphibian fauna in this area was investigated from February 2009 to June 2011 with a total of 10 observation nights. A total of 43 species of amphibians from 24 genera and seven families were recorded in this area including two species of caecilians, *Caudacaecilia larutensis* and *Ichthyophis* sp. This number constitutes 40% out of 107 amphibian species that were found throughout Peninsular Malaysia. Most of the species are from the family of Ranidae (23.3%), followed by Dicroglossidae (18.6%), Microhylidae (16.3%), Rhacophoridae (16.3%), Megophryidae (11.6%), Bufonidae (9.3%) and Ichthyophiidae (4.7%). From this study, the number of amphibian species at Bukit Larut, Perak has increased from 36 to 56 species.

Keywords: Banjaran Bintang, frog species, river, biodiversity, lower part, upper part

INTRODUCTION

Bukit Larut (Maxwell Hill) is the smallest and oldest hill station in Peninsular Malaysia, opened by the British in 1884 as a rest and recreation centre (Malaysia Vacation Guide, 2013). From the foothill, Bukit Larut peak can be reached by walking or using a four-wheeled vehicle provided by the Taiping Municipal Council. The 13 km road to the top of Bukit Larut is very steep and narrow. Along the way, there are many forest streams and temporary puddles that provide suitable habitats for amphibians and reptiles.

Survey on amphibians and reptiles at Bukit Larut was started a century ago. Boulenger (1900) discovered 11 new species, namely, *Leptolalax heteropus, Amolops larutensis, Philautus vermiculatus, Microhyla butleri, M. annectens,*

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E-mail addresses:
shahriza20@yahoo.com (Shahriza Shahrudin),
jibrahim@usm.my (Ibrahim Jaafar)
* Corresponding author

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Hemiphyllodactylus harterti, Draco fimbriatus, D. formosus, Sphenomorphus praesignus, Lygosoma stellatum and *Lycodon butleri*. More recently, many new species, especially lizards have been discovered from Bukit Larut (Hallermann & McGuire, 2001; Grismer *et al*., 2008; Grismer *et al*., 2009; Wood *et al.*, 2009). The most recent faunal list by Grismer *et al*. (2010) reported 36 species of amphibians (34 frogs and two caecilians), and yet some areas remain unexplored.

Many potential areas around Bukit Larut should intensively and systematically be survey to discover more species. Hence, the main objective of this study were to observe and record as many as possible the amphibian species inhabiting Bukit Larut.

MATERIALS AND METHODS

Bukit Larut (4^0 86'N, /100^0 79'E, 1036 m asl) (see Fig.1) is located within the Banjaran Bintang Range. The foothill of Bukit Larut is 4 and 72 km from Taiping and Ipoh, respectively. Several important rivers such as Sungai (Sg.) Kurau, Sg. Bubu, Sg. Batu Tugoh, Sg. Air Terjun and Sg. Ranting arise from Banjaran Bintang around the Bukit Larut areas. Another prominent peak, Gunung Hijau (1448 m asl) is located in the northeast of Bukit Larut. This peak can be reached by following the Gunung Hijau trail that begin near the Telecom communications tower. The vegetations of Bukit Larut range, from lowland dipterocarp forest at the foothill to montane forest at the top.

Study on amphibian fauna of Bukit Larut was conducted in a three-year period starting from February 2009 until Jun 2011 with a total 10 nights of observation (10x consecutive visits, 2 days per visit). Sampling and observation were done at the lower (lowland dipterocarp forests below 300 m asl.) and upper (lower montane forests between 1000 and 1300 m asl.) parts of Bukit Larut. At the lower part, observations were done around the forest trails, forest streams, rivers, puddles and km 1-5 to the top of Bukit Larut. The three main rivers at the foothill (namely, Sg. Batu Tugoh, Sg. Air Terjun and Sg. Ranting) were intensively surveyed for amphibians. Sampling was done along the river transect (500 m) and within areas 3-4 m away from the rivers. At the upper part of Bukit Larut, sampling was done along the roadside (km 9-13), i.e. at a forest stream at km 9, puddles and rainpools at km 11-12 and a small forest stream at km 12.

All the captured specimens were fixed with 10% formalin and stored in 70% ethanol and later deposited at the School of Pharmaceutical Sciences for references. Each collected specimens were photographed before preservation. Berry (1975) and Norhayati *et al.* (2011) were used as reference to identify the amphibian species.

RESULTS

Forty-three species of amphibians (41 frogs and two caecilians) from 24 genera and seven families were confirmed to inhabit Bukit Larut areas. These data constitute 40% of 107 amphibian species found throughout Peninsular Malaysia. Out of

this number, 24 species were found at the lower part (< 300 m asl), 11 species at the upper part (> 1000 m asl) and eight species at both lower and upper parts. The checklist and the number of amphibians observed are presented in Table 1. Ranidae constituted 23.3% of the amphibian species, followed by Dicroglossidae (18.6%), Rhacophoridae (16.3%), Microhylidae (16.3%), Megophryidae (11.6%), Bufonidae (9.3%) and Ichthyophiidae (4.7%). A comparison of the species obtained between Grismer *et al*. (2010) and this study is shown in Table 2.

Species Account

Bufonidae

Ansonia malayana (Inger, 1960) (Fig.2)
Two adult males (11USM-BL-AM01, 02) were caught in February 2011 while calling from a big boulder (app. 1 m above ground) near a small stream (3-4 m width) at km 9 from the foothill.

Duttaphrynus melanostictus (Schneider, 1799)
Four individuals (three adults and one juvenile) were observed near a fish pond and a ditch at the foothill of Bukit Larut. One gravid female (09USM-BL-DM01) was collected in February 2009 as a voucher.

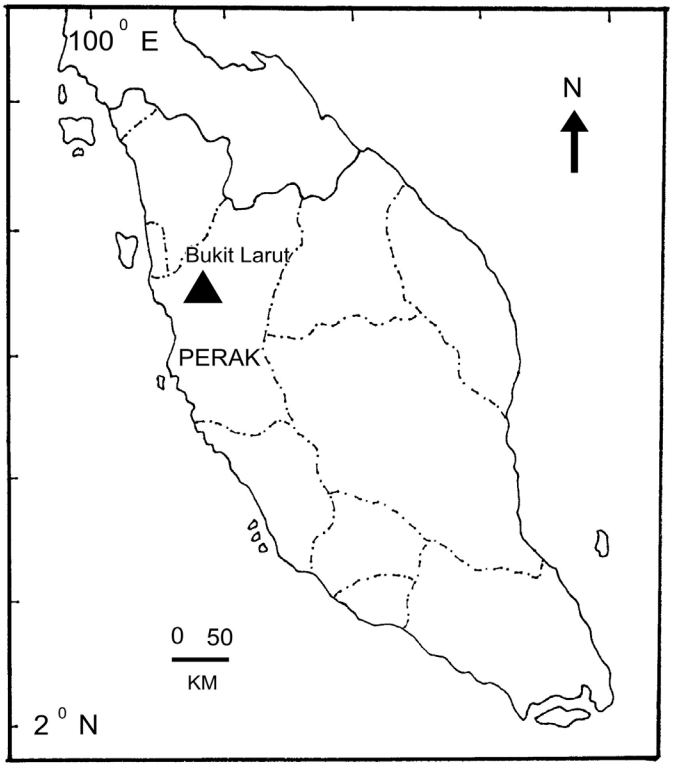

Fig.1: Location of Bukit Larut, Perak

TABLE 1
Amphibian checklist of Bukit Larut, Taiping, Perak

Taxa	Number of individual										
	2009 (lower part, < 300m asl)			2010 (lower part, < 300m asl)			2011 (upper part, > 1000m asl)				
	Feb	Jun	Oct	Jan	May	Sep	Jan	Feb	Apr	Jun	Total
Bufonidae (4)											
Ansonia malayana +	0	0	0	0	0	0	1	2	0	1	4
Duttaphrynus melanostictus	3	1	0	2	2	3	0	0	0	0	11
Ingerophrynus parvus	0	0	2	1	0	2	0	0	0	0	5
Phrynoidis aspera	5	4	7	3	5	4	1	3	2	2	36
Dicroglossidae (8)											
Fejervarya cancrivora	0	0	1	2	1	0	0	0	0	0	4
Fejervarya limnocharis	4	2	3	5	3	2	0	0	0	0	19
Limnonectes blythii	0	1	2	0	1	3	2	3	1	0	13
Limnonectes kuhlii	0	0	1	2	0	1	2	3	2	1	12
Limnonectes laticeps	2	1	0	0	1	3	2	1	1	2	13
Limnonectes malesianus	1	0	0	0	1	1	0	0	0	0	3
Occidozyga laevis	3	0	0	1	2	3	0	0	0	0	9
Occidozyga martensii	1	1	0	0	2	1	0	0	0	0	5
Megophryidae (5)											
Leptobrachium hendricksoni	0	2	2	1	0	2	0	0	0	0	7
Leptolalax heteropus	0	0	0	0	0	0	1	2	1	0	4
Megophrys nasuta	0	2	1	3	1	3	0	0	0	0	10
Xenophrys aceras	0	0	0	0	0	0	0	0	0	1	1
Xenophrys longipes +	0	0	0	0	0	0	1	3	2	1	7
Microhylidae (7)											
Kaluola pulchra	2	1	1	3	0	1	0	0	0	0	8
Metaphrynella pollicaris	0	0	0	0	0	0	0	1	0	2	3
Microhyla berdmorei	0	0	1	0	0	0	0	0	0	0	1
Microhyla butleri +	7	5	3	4	3	6	0	0	0	0	28
Microhyla fissipes	1	0	0	1	0	0	0	0	0	0	2
Microhyla heymonsi	6	7	4	3	4	5	0	0	0	0	29
Phrynella pulchra	0	0	0	0	0	0	0	0	2	0	2
Ranidae (10)											
Amolops larutensis +	8	6	9	7	4	6	3	5	4	3	55
Hylarana banjarana	0	0	0	0	0	0	2	4	3	1	10
Hylarana erythraea	2	1	0	3	2	1	0	0	0	0	9
Hylarana glandulosa	1	4	3	1	2	3	0	0	0	0	14
Hylarana labialis	3	2	1	3	2	4	0	0	0	0	15
Hylarana luctuosa	0	0	0	0	0	1	0	0	0	1	2
Hylarana laterimaculata	0	0	1	1	0	0	0	0	0	0	2
Hylarana nicobariensis	1	0	0	1	0	0	0	0	0	0	2
Hylarana picturata	1	0	0	2	1	0	0	0	0	0	4
Odorrana hosii	2	0	0	3	1	2	0	1	1	0	10
Rhacophoridae (7)											
Nyctixalus pictus	1	0	0	0	1	0	0	0	0	0	2
Philautus parvulus	0	0	1	0	2	1	0	0	0	0	4
Philautus petersi	0	0	0	0	0	0	2	5	4	2	13
Philautus vermiculatus +	0	0	0	0	0	0	3	4	3	2	12
Polypedates leucomystax	1	3	2	1	2	1	1	2	1	0	14
Polypedates macrotis	0	0	1	0	0	1	0	0	0	0	2
Rhacophorus prominanus	0	0	0	0	0	0	1	0	0	0	1
Ichthyophiidae (2)											
Caudacaecilia larutensis +	0	0	0	0	0	0	0	4	2	0	6
Ichthyophis sp.	1	0	0	0	0	0	0	0	0	0	1
Number of individual	56	43	46	54	43	60	22	43	29	19	415
Number of species	21	16	19	23	21	24	13	15	14	12	43

Note: + type locality

TABLE 2
Comparison of amphibian species obtained from Bukit Larut

Taxa	Grismer *et al.* (2010)	This study
Bufonidae		
Ansonia malayana	+	+
Duttaphrynus melanostictus	+	+
Ingerophrynus parvus	–	+
Ingerophrynus quadriporcatus	+	–
Phrynoidis aspera	+	+
Dicroglossidae		
Fejervarya cancrivora	–	+
Fejervarya limnocharis	+	+
Limnonectes blythi	+	+
Limnonectes kuhlii	+	+
Limnonectes laticeps	+	+
Limnonectes malesianus	–	+
Limnonectes plicatellus	+	–
Occidozyga laevis	–	+
Occidozyga martensii	–	+
Megophryidae	+	+
Leptobrachium hendricksoni	–	+
Leptolalax heteropus	+	+
Leptolalax pelodytoides	+	–
Megophrys nasuta	–	+
Xenophrys aceras	+	+
Xenophrys longipes	+	+
Microhylidae		
Chaperina fusca	+	–
Kaloula pulchra	–	+
Metaphrynella pollicaris	+	+
Microhyla annectens	+	–
Microhyla berdmorei	–	+
Microhyla butleri	+	+
Microhyla fissipes	–	+
Microhyla heymonsi	–	+
Phrynella pulchra	+	+

Taxa	Grismer *et al.* (2010)	This study
Ranidae		
Amolops larutensis	+	+
Hylarana banjarana	+	+
Hylarana erythraea	+	+
Hylarana glandulosa	–	+
Hylarana hascheana	+	–
Hylarana labialis	–	+
Hylarana luctuosa	+	+
Hylarana laterimaculata	–	+
Humerana miopus	+	–
Hylarana nicobariensis	–	+
Hylarana nigrovittata	+	–
Hylarana picturata	–	+
Odorrana hosii	+	+
Rhacophoridae		
Nyctixalus pictus	+	+
Philautus parvulus	–	+
Philautus petersi	+	+
Philautus vermiculatus	+	+
Polypedates leucomystax	–	+
Polypedates macrotis	–	+
Rhacophorus bipunctatus	+	–
Rhacophorus cynaopunctatus	+	–
Rhacophorus prominanus	+	+
Theloderma asperum	+	–
Theloderma leprosa	+	–
Ichthyophiidae		
Caudacaecilia larutensis	+	+
Ichthyophis sp.	+	+

Note:
+ = Present
– = Absent

Ingerophrynus parvus (Boulenger, 1887)
Two adult males (09USM-BL-IP01, 02) were captured in October 2009, hiding under dead leaves in the cement ditch along the road to Bukit Larut (km 1.5-2).

Phrynoidis aspera (Gravenhorst, 1829)
This is the Common river toad, which could easily be found at the upper and lower hill. This species was observed in all the months during the sampling period, with two adult males were collected as vouchers. A single specimen (09USM-BL-PA01) was captured in June 2009, while calling from a big rock near Sg. Batu Tugoh (4-5 m width) at the foothill, while the other (11USM-BL-PA01) was captured in January 2011 near a small stream (km 9, 3-4 m width) at upper hill.

Dicroglossidae

Fejervarya cancrivora (Gravenhorst, 1829)
An adult male (09USM-BL-FC01) was caught in October 2009 on the forest floor near Sg. Batu Tugoh at foothill of Bukit Larut.

Fejervarya limnocharis (Gravenhorst, 1829)
Two adult males and one gravid female (10USM-BL-FL01, 02, 03) were collected in May 2010 sitting on the ground at the car park after heavy rain. Another eight specimens, including a single amplectant pair, were observed at the same area.

Limnonectes blythii (Boulenger, 1920)
This species was observed both in lower and upper hill in almost all months. An adult male (09USM-BL-LB01) was captured in June 2009 near Sg. Air Terjun (3-4 m width) at lower part, while another adult male (11USM-BL-LB01) was captured in April 2011 beside a road (km 9.5- 10) at upper part in April 2011.

Limnonectes kuhlii (Tschudi, 1838) (Fig.3)
A single juvenile (09USM-BL-LK01) was caught in October 2009 among leaf litter near Sg. Batu Tugoh, while an adult male (11USM-BL-LK01) was caught in January 2011 in shallow puddles (km 9.5-10) at upper part of Bukit Larut.

Limnonectes laticeps (Boulenger, 1882)
Two adult males (09USM-BL-LL01, 02) were captured in February 2009 perched on the rock at Sg. Ranting (4-5 m width) and another adult male (11USM-BL-LL01) in April 2011 near a small forest stream (km 9) at upper part.

Limnonectes malesianus (Kiew, 1984)
An adult male (10USM-BL-LM01) was collected in May 2010 on forest floor near Sg. Air Terjun.

Occidozyga laevis (Gunther, 1858)
Two adult males and a single adult female (09USM-BL-OL01, 02, 03) were caught in February 2009 in small water puddles near Sg. Batu Tugoh. An amplectant pair was also observed at the same site but we did not collected it.

Occidozyga martensii (Peters, 1867)
An adult male (09USM-BL-OM01) was captured in June 2009 hiding under a dead wood near Sg. Air Terjun.

Megophryidae

Leptobrachium hendricksoni (Taylor, 1962)
Two adult males (09USM-BL-LHen01, 02) were collected in Jun 2009 among the leaf litter in a cement drain at km 0.5-1 after evening rain. Their tadpoles were also observed in Sg. Batu Tugoh.

Leptolalax heteropus (Boulenger, 1900)
A single adult was observed in January 2011 perched on dead woods near a small forest stream (km 9) at upper part.

Megophrys nasuta (Schlegel, 1858)
Two adult males (09USM-BL-MN01, 02) were captured in Jun 2009 while calling under a big rock and a wood near a small forest stream (km 0.5-1) along the way to the peak of Bukit Larut. Several other specimens were heard calling at the same area in the late evening.

Xenophrys aceras (Boulenger, 1903) (Fig.4)
A single adult male (11USM-BL-XA01) was collected in Jun 2011 perched on a wet rock near a small forest stream (km 11.5-12) at the upper part after rain.

Xenophrys longipes (Boulenger, 1886) (Fig.5)
A single juvenile (11USM-BL-XL01) was caught in January 2011 perched on a mossy rock near a forest stream (km 9) while a gravid female (11USM-BL-XL02) was caught in Jun 2011 beside the road (km10-10.5) at the upper part after evening rain.

Microhylidae

Kaloula pulchra (Gray, 1831)
An amplectant pair (09USM-BL-KP01, 02) was captured in February 2009 in a ditch near the toilet at the foothill of Bukit Larut after heavy rain. Several calling males were also heard around the area.

Metaphrynella pollicaris (Boulenger, 1890)
An adult male was observed in February 2011 calling from the tree branches (app. 1.5 m above ground) at upper part (km 10.5-11). Several other males (6-8 individuals) were heard calling from tree branches (1-2.5 m above ground) at the same area.

Microhyla berdmorei (Blyth, 1856) (Fig.6)
An adult was collected (09USM-BL-MBer01) in October 2009 on the forest floor near a small forest stream (km 0.5-1) along the way to the peak of Bukit Larut.

Microhyla butleri (Boulenger, 1900)
This species was found in all months at the lower part but did not found it at the upper part of Bukit Larut. Two adult males (10USM-BL-MBut01, 02) were collected in September 2010 among the leaf litter at the foothill after evening rain. An amplectant pair was also found at the same site but we did not collect it.

Microhyla fissipes (Boulenger, 1884)
An adult (09USM-BL-MF01) was collected in February 2009 hiding among the leaf litter near Sg. Air Terjun.

Microhyla heymonsi (Vogt, 1911)
This species was also found in all months at the lower part but did not found at the upper part. Four specimens (09USM-BL-MH01, 02, 03, 04) including three adult males and one adult female were captured in October 2009 hiding under the tall grass and leaf litter at the foothill. Several other individuals (6-8 males) were actively calling from the shrubs at the same site.

Phrynella pulchra (Boulenger, 1887) (Fig.7)
Two adult specimens (11USM-BL-PP01, 02) were collected in April 2011 perched on fern tree (app. 1 m above ground), while another in a cement drain (km 11-11.5) along the way to the peak of Bukit Larut. Both of the specimens were not calling then and were collected after evening rain.

Ranidae

Amolops larutensis (Boulenger, 1899)
This species was found in all months, both at upper and lower part. Two adult males (09USM-BL-AL01, 02) were caught in February 2009 perched on a big boulder at Sg. Ranting and another two juveniles (11USM-BL-AL01, 02) were caught in June 2011 sitting on a wet rock in a forest stream (km 9) at upper part. Several other specimens (6-10 individuals) were found at both sites but we did not collect them.

Hylarana banjarana (Leong & Lim, 2003) (Fig.8)
Two adult males (11USM-BL-HB01, 02) were captured in February 2011. One specimen was captured sitting on a mossy rock near a forest stream (km 9), while another one in the wet cement drain (km 11-11.5) at the upper part. Both specimens were actively calling when captured.

Hylarana erythraea (Schlegel, 1837)
Four specimens were observed near a fish pond at the foothill but only a single gravid female (09USM-BL-HE01) was collected in June 2009 as a voucher.

Hylarana glandulosa (Boulenger, 1882)
An adult male (10USM-BL-HG01) was captured in January 2010 near a small forest stream at the foothill. Several other males were actively calling at the same sites.

Hylarana labialis (Boulenger, 1887)
Three specimens (09USM-BL-HLab01, 02, 03) including two adult males and one adult female were caught in February 2009 perched on tree branches and wet rock near Sg. Ranting. Several other specimens were also observed at Sg. Air Terjun.

Hylarana luctuosa (Peters, 1871) (Fig.9)
An adult male (11USM-BL-HLuc01) was collected in June 2011 among the leaf litter in a wet cement drain (km 11-11.5) at upper part after raining.

Hylarana laterimaculata (Barbour and Noble, 1916)
One adult male (10USM-BL-HLat01) was captured in January 2010 while calling from a fern tree (0.5 m above ground) at the river bank of Sg. Batu Tugoh.

Fig.2: *Ansonia malayana*

Fig.3: *Limnonectes kuhlii*

Fig.4: *Xenophrys aceras*

Fig.5: *Xenophrys longipes*

Fig.6: *Microhyla berdmorei*

Fig.7: *Phrynella pulchra*

Fig.8: *Hylarana banjarana*

Fig.9: *Hylarana luctuosa*

Hylarana nicobariensis (Stoliczka, 1870)
A single adult male (09USM-BL-HN01) was captured in February 2009 in a puddle near Sg. Air Terjun.

Hylarana picturata (Boulenger, 1920)
Two adult males were captured. One specimen (09USM-BL-HP01) was caught in February 2009 perched on a dead twig near Sg. Ranting and another specimen (10USM-BL-HP01) was caught in January 2010 on the forest floor near Sg. Air Terjun. Both specimens were actively calling when captured.

Odorrana hosii (Boulenger, 1891)
This species was found both at the lower and upper parts. An adult male (10USM-BL-OH01) was collected in May 2010 perched on a big boulder at Sg. Batu Tugoh and another adult female (11USM-BL-OH01) was collected in February 2011 sitting on a wet rock near a forest stream (km 9) at the upper part.

Rhacophoridae

Nyctixalus pictus (Peters, 1971)
A juvenile was observed in February 2009 perched on tree leaves (0.5 m above ground) near a small puddle at the foothill.

Philautus parvulus (Boulenger, 1893)
An adult (09USM-BL-PPar01) was collected in October 2009 perched on a fern tree near a small stream (km 0.5-1) after evening raining.

Philautus petersi (Boulenger, 1900) (Fig. 10)
Two adults (11USM-BL-PPet01, 02) including a male and female were caught in February 2011 perched on a fern tree (app. 2 m above ground, km 9.5-10) and another two adult males (11USM-BL-PPet03, 04) were caught in Jun 2011 perched on tree leaves (app. 1-2 m above ground, km 10.5-11) at upper part. An amplectant pair and several calling males were also observed at the same area.

Philautus vermiculatus (Boulenger, 1900)
Two adult males (11USM-BL-PV01, 02) were captured in January 2011, perched on leaves and fern tree (app. 1.5-2 m above ground, km 11.5-12) at the upper part after evening rain. Several other males (5-7 individuals) were actively calling at the same area.

Polypedates leucomystax (Gravenhorst, 1829)
This species was found both at the lower and upper parts. We collected two specimens (11USM-BL-PL01, 02) including a male and a gravid female in February 2011 near a fish pond (Beringin bungalow) at the upper part. Two foam nests were also observed at the same site.

Polypedates macrotis (Boulenger, 1891)
An adult male (09USM-BL-PM01) was caught in October 2009 perching on a dead twig near the puddles at the foothill.

Rhacophorus prominanus (Smith, 1924)
An adult (11USM-BL-RP01) was captured in January 2011 perched on leaves (app. 2 m above ground) near a forest stream (km 9) at the upper part after evening rain.

Ichthyophiidae

Caudacaecilia larutensis **(**Taylor, 1960) (Fig.11)
One juvenile (11USM-BL-CL01) was collected in April 2011 in a wet cement drain accumulated with dead leaves (km 9) at the upper part after rain.

Ichthyophis sp. (Taylor, 1960)
A juvenile specimen (09USM-BL-Csp01) was collected in February 2009 in the puddles accumulated with dead leaves near Sg. Batu Tugoh. This specimen has a yellow stripe on both sides of the body.

DISCUSSION

The pristine and undisturbed forest of Bukit Larut is very rich with amphibian and reptile species. A previous study by Grismer *et al*. (2010) reported 36 species of amphibians at Bukit Larut, although their study was focused on species of higher elevations (Bukit Larut at 800-1300 m and Gunung Hijau Trail at 1200-1448 m asl). In this study, both the lower (< 300 m asl) and upper (1000-1300 m asl) parts of Bukit Larut were covered, and more amphibian species were discovered from the lower altitudes. Twenty species of amphibians from the lowland forest (< 300 m asl) were added to the list prepared by Grismer *et al.* (2010), increasing the number of amphibian species at Bukit Larut from 36 to 56.

The amphibian species from the lower part of Bukit Larut included *I. parvus* from Bufonidae, *F. cancrivora, L. malesianus, O. laevis* and *O. martensii* from Dicroglossidae, *L. hendricksoni* and *M. nasuta* from Megophryidae, *K. pulchra, M. berdmorei, M. fissipes* and *M. heymonsi* from Microhylidae, *H. glandulosa, H. labialis, H. laterimaculata, H. nicobariensis* and *H. picturata* from Ranidae, *P. parvulus, P. leucomystax* and *P. macrotis* from Rhacophoridae, and *Ichthyophis* sp. from Ichthyophiidae. All these species were found only at the lower part, except for *P. leucomystax,* which was also found at the

Fig.10: *Philautus petersi*

Fig.11: *Caudacaecilia larutensis*

upper hill. Generally, the species mentioned above are lowland species and can easily be found at an altitude below 300 m asl. Previous studies by Ibrahim *et al.* (2012a,b) at Sg. Sedim (compartment 15) and Bukit Perangin and Shahriza *et al*. (2011) at Bukit Hijau also recorded the occurrence almost of these species at elevation 100, 105 and 300 m asl. However, other studies recorded some of these species at higher elevation. For example, Norhayati *et al.* (2011) found two species of frogs, *M. nasuta* and *H. labialis* at Fraser hill (1448 m asl). The lower part, especially at the foothill area is more disturbed, and three commensal species that associated with human, *K. pulchra, M. heymosi* and *P. leucomystax* are easily to find. Other, are typical forest frogs that inhabit in the lowland forest and need more specific and clean environments that cannot be found in the disturbed areas.

Bukit Larut is the type locality for several species of frogs, such as *Ansonia malayana, Xenophrys longipes, Microhyla butleri, Amolops larutensis, Philautus vermiculatus* and *Caudacaecilia larutensis* (Grismer *et al.,* 2010). In this survey, we could find all of these species, which indicated that these species have survived in Bukit Larut forest for a long time.

Observation and sampling at Bukit Larut were done around easily accessed areas and did not covered the areas deep in the forest such as old forest trails, forest floors, tree canopy, forest streams, swamps and waterfalls because of the lacking of manpower and time. In the future, there is a need to explore more undisturbed areas to get the overall picture of the occurrence of amphibians at Bukit Larut.

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Carcass and Meat Characteristics of Traditionally Managed Nigerian Yankasa and West African Dwarf Breeds of Sheep

Fasae O. A.[1]*, Oduguwa B. O.[2], Adejumo L. A.[1] Makinde T. E.[1] and Sanwo K. A.[1]

[1]Department of Animal Production and Health, Federal University of Agriculture, P.M.B. 2240, Abeokuta, Nigeria
[2]Institute of Food Security, Environmental Resources and Agricultural Research, Federal University of Agriculture, P.M.B. 2240, Abeokuta, Nigeria

ABSTRACT

The carcass characteristics of Nigerian West African dwarf (WAD) and Yankasa breeds of sheep managed under traditional systems were compared. These two breeds were represented by six animals each with an average body weight of 18.97±0.36kg. The animals were slaughtered and carcass weight, yields, composition and sensory properties scores were recorded. Results indicated that breed primarily differed in carcass traits and retail cuts. Yankasa breed had a significant higher ($P<0.05$) empty body weight, cold carcass weight. Dressing percentage varied ($P<0.05$) from 46.38 and 48.60% for WAD and Yankasa breeds, respectively. The loin and leg cuts were statistically ($P>0.05$) ranked the same across the two breed. Weights of the head and empty gut were significantly ($P<0.05$) lower in WAD sheep compared with Yankasa sheep. The crude protein and fat contents of meat from loins were higher ($P<0.05$) in WAD sheep. Following assessment, eating quality traits varied across breeds, panellist-rated flavour, juiciness and overall acceptability higher ($P<0.05$) in WAD meat. In conclusion, Yankasa sheep have a better carcass and dressing percentage but the overall sensory is better for the West African Dwarf. These may be due to the higher fat and protein contents resulted in more flavour, tender and juicy meat.

Keywords: Sheep, Yankasa, West African Dwarf, traditional management, carcass, sensory

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E-mail addresses:
animalexp@yahoo.co.uk (Fasae O. A.),
oduguwa2002@yahoo.com (Oduguwa B. O.),
bumogardens@yahoo.com (Sanwo K. A.)
* Corresponding author

INTRODUCTION

In Nigeria, sheep is reared traditionally at subsistence level. They are mostly managed semi-intensively; usually left to scavenge and cater for their own nourishment with domestic leftovers whose composition

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depends on the family menu that may constitute part of sheep's diet (Fasae *et al.,* 2012). These animals are mostly reared for meat and are also important sources of milk, skins and manure. Their fecundity, short generation interval and capability for fitting into all existing agricultural production systems, as well as the prevailing demand for mutton, place them in a unique position (Ozung *et al.,* 2011). Sheep production in south western Nigeria is centred on the Yankasa and West African dwarf breeds. Compared to other breeds found in Nigeria, these breeds of sheep are classified in the lightweight sheep category with slaughter weight range of 15-30 kg. An understanding of carcass characteristics and meat quality traits of these sheep breeds is important because of the butcher's expectation and consumer demand for superior quality meat with optimum food value as well as the importance of protein in human diet cannot be over-emphasized.

This paper compares the carcass characteristics, chemical composition, and sensory evaluation of two common Nigerian breeds of sheep (Yankasa and West African dwarf) managed under the semi intensive system.

MATERIALS AND METHODS

Research Location

The study was conducted in the Teaching and Research Farms and the meat processing laboratory of the Animal Production and Health Department, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria.

Experimental Animals and Their Management

Twelve (12) rams made up of six (6) Yankasa and six (6) West African dwarf (WAD) breeds with age range of 18-24 months and an average weight of 18.97±0.36kg, and managed semi-intensively were sourced from smallholder sheep farmers in Odeda, the local government area of Ogun state in the southwest of Nigeria. The vegetation around the area of purchase is luxuriant with *Pennisetum purpureum* and *Panicum maximum*. Other species of grasses and legumes available are *Andropogon tectorum, Imperata cylindrica, Centrosema pubescens, Stylosanthes hamata, Calapogonium mucunoides, Pueraria phaseoloides* and *Gliricidia sepium.* The sheep move out to graze early in the morning and come back around 1700hrs in the afternoon when they are given crop residues and household wastes as supplement in sheds. A spring balance scale was used to measure the weight of the sheep at purchase while the dentition method (Abegaz & Awgichew, 2009) was used to ascertain the age of the animals. The animals were allocated to two treatment groups according to their respective breeds.

Slaughter and Carcass Dissection

Prior to the slaughtering processes, the animal were fasted for 20 hours with free access to water and slaughtered according to the local method by severing the jugular vein and the carotid arteries. After thorough bleeding, the hair was scalded from the skin using boiling water as described by

Fasae *et al.* (2011). Empty body weight was calculated by subtracting the weight of the gut content from the slaughter weight. The hot carcass weight was the weight after removing the head, feet and gastric intestinal tract within one hour of slaughter. The internal organs (kidney, liver, heart and spleen) were carefully excised and weighed. The cold carcass was the weight after 24 hours in a freezer at 4^{o}C. The carcasses were cut into retail parts (shoulder, rack, loin, legs, neck/breast, and shank/flank) and each part was weighed according to the method described by Adu and Brinckman (1981). The dressing percentage was calculated as the ratio of cold carcass weight to live weight in percentage.

Sensory Evaluation

In evaluating the sensory qualities, samples of meat from each group were collected from the loin area, cut into chops of an average weight of 150g and cooked in water at a temperature of 65^{o}C for 30 minutes in a pot using a gas cooker. They were then coded and the serving sequence was randomized. Ten panellists were trained in the assessment procedure two days prior to the evaluation and were subsequently required to masticate on a sample from each treatment and score it for flavour, tenderness, juiciness and overall degree of acceptability. Water was served to the panellist to rinse their mouths after scoring each sample to minimize flavour carry over.

The evaluators scored each sample on a nine (9) point hedonic scale (9 = like extremely enjoy; 1 = extremely disliked) (Pena *et al.,* 2009) for colour, juiciness, flavour, texture, tenderness. Overall acceptability was scored on a 3-point scale (1 = least acceptable; 2 = more acceptable and 3 = most acceptable) (Iwe, 2002). Cooking loss was determined as the difference between pre-cooked and post-cooked weights, divided by pre-cooked weight of meat and multiplied by 100.

For the chemical composition of the meat, about 50g of meat cuts from the loin was kept at -20^{o}C until required for chemical analysis. The crude protein, fat (ether extract) ash and DM contents were determined by using the Kjeldahl method, soxhlet extraction, oven drying and burning the samples in the electric furnace, respectively (AOAC, 1995).

STATISTICAL ANALYSIS

The data obtained were subjected to analysis using the T-test (SAS, 1999).

RESULTS AND DISCUSSION

The comparison of the carcass characteristics of Yankasa and WAD sheep is shown in Table 1. The results revealed that carcass components observed differed significantly ($P<0.05$) across the breeds with WAD sheep having significantly ($P<0.05$) lower values compared to Yankasa sheep. The higher carcass weight observed in Yankasa sheep could be attributed by the weight at slaughter, and this agrees with the findings of Lawrie (1998) that carcass composition is weight dependent and largely uninfluenced by age or nutritional regime.

The carcass yields of Yankasa and WAD sheep in this study are lower compared with those reported for some tropical sheep (Adu & Brinckman, 1981; Madhavi *et al.,* 2006; Kawas *et al.,* 2007). This could be as a result of breed differences in this study. The dressing percentage (DP) of Yankasa sheep (48.60%) was higher (P<0.05) than WAD sheep (46.38%). Meanwhile, DP observed for WAD sheep in this study is lower to those reported by Alkoriet *et al.* (2007) and Fasae *et al.* (2011) for the same breed of sheep under zero grazing. Moreover, DP obtained for Yankasa sheep in this study is lower than those earlier reports of Adu and Brinckman (1981) for Yankasa and crossbred sheep, which could be attributable to the breed, management system and nutrition. However, these values are comparable to 44% and 48% DP in Omani sheep (Mahgoub *et al.,* 2000) and crossbreds of Mexican Pelibuey with Rambouillet sheep (Guttierez *et al.,* 2005), respectively.

Regardless of the carcass weight, the distribution of non-carcass components seemed to be similar (P>0.05) across the two breeds with exception of the head and empty gut weights, which were significantly (P<0.05) lower in WAD than the Yankasa sheep. Higher values were obtained for the WAD sheep (Fasae *et al.,* 2011) and the relationships between the physical component of carcass cuts and whole carcass have been shown in several studies (El Karim *et al*., 1988; Cameron, 1992).

Table 2 shows the comparison of the weight and percentages of retail cuts from WAD and Yankasa sheep. The leg was not affected (P>0.05) by breed. The loin, neck/ breast and shank/flank differed across the

TABLE 1
Carcass characteristics of Yankasa and West African dwarf sheep

Components	Yankasa sheep	West African Dwarf
Slaughtered weight (kg)	$20.33^{a} \pm 0.41$	$17.83^{b} \pm 0.36$
Empty body weight (kg)	$17.87^{a} \pm 0.37$	$15.04^{b} \pm 0.29$
Hot carcass weight (kg)	$12.30^{a} \pm 0.44$	$10.39^{b} \pm 0.12$
Cold carcass weight (kg)	$9.88^{a} \pm 0.09$	$8.27^{b} \pm 0.08$
Dressing percentage (%)	$48.60^{a} \pm 0.77$	$46.38^{b} \pm 0.65$
Non-carcass components (kg)		
Head	$8.91^{a} \pm 0.02$	$7.37^{b} \pm 0.32$
Heart	0.56 ± 0.01	0.55 ± 0.03
Kidney	0.37 ± 0.21	0.33 ± 0.02
Lung/Trachea	1.16 ± 0.31	1.13 ± 0.03
Liver	1.27 ± 0.11	1.31 ± 0.04
Spleen	0.28 ± 0.03	0.33 ± 0.01
Testes	0.93 ± 0.05	0.89 ± 0.02
Empty gut	$9.93^{a} \pm 0.41$	$8.20^{b} \pm 0.03$
Drainable blood	6.72 ± 0.32	7.09 ± 0.11

[a,b] mean values in the same row with the same superscripts are not significantly different (P>0.05)

breeds with WAD sheep having significantly (P<0.05) lower values compared to Yankasa sheep. The leg and loin cuts of the experimental sheep were not affected (P>0.05) by breed, which corroborates with the findings of Reddy and Reddy (2001). The shank/flank and rack cuts of Yankasa sheep were significantly higher (P<0.05) than WAD sheep. Nonetheless, significant (P>0.05) difference was not observed in the distribution of meat and bone in the leg of the animals.

The fairly close meat to bone ratio of rams in this experiment is similar to those reported by Adu and Brinckman (1981), which is probably an indication of uniform rate of meat and bone deposition in the experimental rams. The bone in leg and loin cuts which contributed to 32% to 33% of the total weight across the breed are similar to the range reported by Adu and Brinckman (1981), but higher than 27 and 24% reported in Menz than Horro sheep, respectively (Ewnetu *et al.,* 2006).

The proximate composition of meat from loin collected from Yankasa and WAD sheep is shown in Table 3. There is no significant (P>0.05) difference observed in the dry matter (DM) contents suggesting that the DM of meat across the breeds is closely

TABLE 2
Mean weights (Kg) of retail cuts of Yankasa and West African dwarf sheep

Components (Kg)	Yankasa sheep	West African Dwarf
Leg	5.43 ± 0.06	5.98 ± 0.02
Loin	6.49 ± 0.07	6.32 ± 0.03
Shoulder	27.07 ± 0.10	26.39 ± 0.81
Rack	30.72[a] ± 0.05	28.09[b] ± 0.95
Neck/Breast	2.13 ± 0.03	1.99 ± 0.01
Shank/flank	3.56[a] ± 0.05	2.39[b] ± 0.03
Leg and loin cuts		
Total weight	2.13± 0.06	1.85± 0.05
Total meat	1.60± 0.04	1.40± 0.03
Total bone	0.53± 0.01	0.45± 0.01
Meat to bone ratio	0.33± 0.02	0.32± 0.01

[a,b] Mean values in the same row with the same superscripts are not significantly different (P>0.05)

TABLE 3
Chemical composition (%) of meat from loin of Yankasa and West African dwarf sheep

Parameters	Yankasa	West African Dwarf
Dry matter	26.73 ± 1.72	27.22 ±1.69
Crude protein	33.50[b] ± 1.95	35.50[a] ±2.05
Fat	7.43[b] ± 0.11	10.20[a] ± 0.16
Ash	4.12 ± 0.05	4.50 ± 0.06

[a, b] Means in the same row with the same superscripts are not significantly different (P>0.05).

related. In contrast, crude protein content ($P<0.05$) varied across the breeds. Also, WAD sheep had higher ($P<0.05$) fat content compared to Yankasa sheep, while the ash content was not affected ($P>0.05$) by breed.

Table 4 shows the mean values of the sensory properties of Yankasa and WAD sheep. Following sensory assessment, there were significant differences ($P<0.05$) observed for eating quality traits. The panellists rated meat from WAD sheep higher ($P<0.05$) in flavour juiciness, tenderness and overall acceptability ($P<0.05$) relative to that of Yankasa meat. This corroborates the findings by Schönfeldt *et al.* (1993) that reported with increasing fatness of carcasses, the tenderness and flavour of the cooked cuts of sheep meat increased significantly. Also, research reports show that flavour ratings appear to be largely related to the panellist's preference and previous exposure to lamb (Sanudo *et al*., 2000). Risvik (1994) indicated that texture and juicy meat is generally preferred by consumers. Moreover, there is a perfect relationship in meat colour from both breeds as rated by the panellists.

CONCLUSION

Based on the results of this study, it could be concluded that Yankasa and West African Dwarf breeds of sheep are to be more considered as being important sources of animal protein. The Yankasa sheep have better carcass and dressing percentages but the overall sensory was better for the West African Dwarf. This may be due to the higher fat and protein contents resulted in more flavour, tender and juicy meat. It is therefore suggested that an improvement in nutrition and breeding programmes of these traditionally managed sheep may possibly produce well-conformed and heavier carcasses, with no apparent detrimental effects on the carcasses quality in the breeds studied.

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TABLE 4
Effect of breed on the sensory properties of meat from loin of Yankasa and West African dwarf sheep

Parameters	Yankasa	West African Dwarf
Flavour	$6.50^{b} \pm 0.24$	$7.38^{a} \pm 0.31$
Tenderness	$6.50^{b} \pm 0.29$	$7.25^{a} \pm 0.34$
Texture	7.00 ± 0.31	7.25 ± 0.28
Colour	7.50 ± 0.43	7.50 ± 0.43
Juiciness	$6.75^{b} \pm 0.32$	$7.25^{a} \pm 0.33$
Acceptability	$7.63^{b} \pm 0.47$	$8.00^{a} \pm 0.42$

^{a,b} Means in the same row with the same superscripts are not significantly different ($P>0.05$).

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The Bacterial Community of the Chicken's Intestinal Tract: Impact of Xylanase Supplement

Samsudin, A. A.[1,2]* and Al-Hassani, D. H.[1,3]

[1]*School of Agriculture and Food Sciences, The University of Queensland, Gatton 4343, Australia*
[2]*Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[3]*Department of Animal Resources, College of Agriculture, Baghdad University, Abu-Ghraib, Baghdad, Iraq*

ABSTRACT

The effect of xylanase supplementation on the profile of the bacterial community of the gastrointestinal tract of layer chicken was investigated using PCR-DGGE analysis. Thirty four common bands, belonging to *Lactobacillus acidophilus* and *Lactobacillus salivarius*, were excised from the DGGE gel, amplified, cloned and sequenced. Sequence analysis of the clones revealed that 79.4% of sequences from the intestine were related to those of *Lactobacillus* spp., while the remaining 20.6% belonged to the four families, *Clostridiaceae* (8.8%), *Streptococcus* (5.8%), *Bacteroides* (3%) and *Enterococcus* (3%). Bacteria belonging to the genus *Lactobacillus* spp. were the predominant bacteria across the different treatments and segments of the gastrointestinal tract of chicken.

Keywords: Bacterial community, Denaturing Gradient Gel Electrophoresis (DGGE), intestinal tract, xylanase

INTRODUCTION

Dietary enzymes supplementation has been widely used in poultry diets in attempts to improve nutrient utilization, health, quality improvement of the product and reduce pollution, as well as to increase the choice of content of ingredients which are acceptable for inclusion in diets (Elmenaway *et al.,* 2010). Apart from that, it was added to the poultry diet to improve the nutritional value of feeds and gut microflora of birds (Anjum & Chaudhry, 2010). Exogenous enzymes have been available for many years, and their use in animal diet has increased exponentially, mainly due to increasing cost of feeding. It can be used to reduce the negative impacts of some feed constituents such as non-starch polysaccharides (NSP) on gut conditions and overall utilisation of dietary energy and proteins (Choct *et al.,*

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E-mail addresses:
anjas@upm.edu.my (Samsudin, A. A.),
alhassani.dihya@yahoo.com (Al-Hassani, D. H.)
* Corresponding author

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2006). The adverse effects of soluble NSP on nutrient digestion and absorption in poultry are due to the ability of the soluble NSP to increase the viscosity of the digesta. Apart from that, it can also modify the physiology and the ecosystems of the gastrointestinal tract (Choct, 2002). High gut viscosity can lead to poor bird performance. It is thought that soluble NSP acts together with the glycocalyx of the intestinal brush border and thickens the rate-limiting unstirred water layer of the mucosa, decreasing the efficiency of nutrient absorption through the intestinal wall (Johnson & Gee, 1981).

The addition of feed enzymes such as xylanase in poultry diets could cleave the large molecules of NSP into smaller polymers, thereby reducing digesta viscosity and increasing the nutritive value of the feed (Bedford *et al.,* 1991; Choct & Annison, 1992). The use of xylanase has shown not only to improve energy utilisation of wheat based chicken diet but also eliminate *Clostridium perfringens* in broiler chicken fed low apparent metabolizable energy (AME) diet (Choct *et al.,* 2006). Supplementation of xylanase in poultry diet seems to decrease the microflora fermentation in the small intestine but increase in the large intestine and ceaca (Steenfeldt *et al.,* 1998). Apart from that, the caecal content pH also decreases due to high production of short-chain fatty acids caused by an increase of microbial fermentation. The increase of the fermentation was indicated by degradation of cell wall arabinoxylans in the enzyme-supplemented diets that increases the amount of material available for microbial fermentation in the ceaca (Steenfeldt *et al.,* 1998). A few studies have been reported that xylanase supplement could change the development of gut microflora, including those attached to the mucosa using microbial culturing techniques (Bjerrum *et al.,* 2006; Hubener *et al.,* 2002; Vahjen *et al.,* 1998). However, the effects of xylanase alone on the bacterial population in the intestinal tract of poultry using molecular culture-independent techniques are less likely to have been reported since most of the published works are actually involved a combination of a few exogenous enzymes (Bjerrum *et al.,* 2006; Torok *et al.,* 2008).

The present work was designed to particularly determine the influence of xylanase on the development of selected groups of intestinal bacterial community of chickens. An understanding of the bacterial population in the intestinal tract of the chickens will allow us to detect changes in the flora and to analyze the effects of food animal management changes. The information presented in this paper may permit us to manipulate intestinal flora with the intention of enhancing intestinal health and feed conversion. Independent molecular techniques were used to examine the bacterial profile in the different segments of the intestines, while the identification of the common bacterial groups was based on the 16S rRNA gene sequence.

MATERIALS AND METHODS

Birds and Their Management

A total of 36, twenty-seven-week old, ISA Brown layers were raised for a period of five weeks before being euthanized for digesta collection. This study was conducted according to the animal ethics guidelines set by the Animal Ethics Committee of the University of Queensland (AEC Approval Number: SAS/372/08/eirdc/fei) in 2010. At the beginning of the experiments, the birds were randomly divided into two equal treatment groups, with 6 replicates (cages) of 3 birds per replicate each. The birds were fed with a basal diet *ad libitum* that did not contain animal protein, growth-promoting antibiotics or coccidiostats. The birds were randomly assigned to either control group (basal diet with no enzyme added, T1), and basal diet + xylanase (T2) (2,500U/kg diet, Sigma-Aldrich, Selangor, Malaysia). The enzyme was added according to the recommendations of the manufacturers. Each basal diet contains wheat, soybean meal and ground corn as major ingredients to provide 11.28 MJ, ME/kg, 15.53% crude protein, 0.5% methionine plus cyctine, 0.7% available lysine and 3.5% calcium. The composition of the basal experimental wheat based diet is shown in Table 1.

Collection of the Samples

At the end of the five weeks feeding period, all the birds were euthanized, while the intestine and caeca contents were removed according to the procedure by Yang *et al.* (2008). Briefly, the small intestine was divided into three segments: duodenum (from the gizzard outlet to the end of the pancreatic loop), jejunum (from the pancreatic loop to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the caecal junction). The contents of the small intestine and large intestine were aseptically flushed with ice-cold phosphate buffered saline (PBS) at pH 7.4 into sterile

TABLE 1
The basal commercial experimental wheat-based diet

Ingredients	g kg^{-1}
Wheat	670.0
Soybean meal	191.0
Vegetable Oil	28.0
Corn meal	83.0
Di-calcium Phosphate	18.0
Salt	3.0
Vitamin-trace mineral premix[a]	7.0

[a]Contents per kg premix: 4.6 mg trans-retinol; 122.5 µg cholecalciferol; 28 mg DL-α-tocopheryl acetate; 2.8 mg menadione; 2.1 mg thiamine; 11.2 mg riboflavin; 21 mg calcium pantothenate, 42 mg niacin; 7 mg pyridoxine; 2.8 mg folic acid; 21 µg cyanocobalamine; 140 µg biotin; 105 mg Mn; 70 mg Zn; 7 mg Cu; 2.2 mg Mo; 420 µg Co; 1.4 mg I; 28 mg Fe; 140 µg Se; 420 mg choline chloride; 175 mg ethoxyquin.

containers, placed over ice and transported immediately to the laboratory for bacterial analysis.

Culture-independent Techniques

Genomic DNA was extracted from the contents of the different segments of the intestines using the non-ionic detergent cetyltrimethylammonium bromide (CTAB) with beads beating, as described by Wright *et al.* (1997). Briefly, approximately 1 gram of the digesta samples were centrifuged (12,000 ×g) for 5 min and the supernatant was removed. Then, 200 mg of silica/ zirconium beads were added and the pellet was resuspended in 800 µl CTAB isolation buffer (100mM Tris-HCl, pH8; 1.4M NaCl; 20mM EDTA (sodium salt); 2% hexadecetyltrimethylammonium bromide). The samples were homogenised in a bead beater for 2 min, cooled on ice and homogenised for another 2 min. The samples were then incubated at 70°C for 20 min, followed by centrifugation at 10,000 g for 10 min. The aqueous phase was transferred to a new microfuge tube and 500 µl phenol/ chloroform/ isoamyl alcohol (25:24:1) were added, vortexed, and centrifuged at 13,000 g for 10 min. Again, 500 µl of the upper aqueous layer was removed and dispensed into another micro-centrifuge tube. DNA was precipitated with 300 µl of isopropanol at room temperature for 5 min. DNA was collected by centrifugation at top speed (14,000 g) for 15 min and the nucleic acid pellet was washed with 1 ml 70% ice-cold ethanol. The pellet was incubated at 70°C for 10 min before centrifuging again at 14,000 g for 10 min. The ethanol was removed and the pellet was air-dried before resuspending it in 50 µl of DNA/RNA free water. DNA from the extracted samples was quantified using a nanodrop spectrophotometer (BioSpec-nano Shimadzu, Queensland, Australia) to check for the quantity and quality of DNA.

The variable V3 region of 16S rDNA was enzymatically amplified in the PCR with primers to conserve regions of the 16S rRNA genes. The nucleotide sequences of the primers are as follows: 341f-GC (5'-CCTACGGGAGGCAGCAG-3'); and 534r (5'-ATTACCGCG GCTGCTGG-3'). Primer 341f has an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5' end. PCR amplification was performed with a MJ Research PTC-100 thermal cycle machine (MJ Research, Inc., Watertown, Mass.). A 50 µl volume PCR reaction contained with 5 µl 10x PCR buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 3 µl 50 mM $MgCl_2$, 1 µl 10 mM dNTPs (Qiagen, Victoria, Australia), 1 µl 10 µM of each primer, 0.3 µl of 5 U/µl of Platinum *Tag* DNA polymerase (Invitrogen, Carlsbad, NM, USA), 250 ng of genomic DNA and DNA/RNA free water to 50 µl. The samples were first incubated for 5 min at 94°C to denature the template DNA. This hot start technique was performed to minimize non-specific annealing primers to non target DNA. The initial hot-start was then followed by 15 cycles of the following parameters: 94°C for 30 s, 65°C for 30 s, and 68°C for 1 min. At the temperature of 65°C, the temperature was decreased by 1°C every second cycle until a touchdown at 55°C was accomplished. Another set of annealing

parameters were as follows: 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, and it was run for 20 cycles. This procedure reduces the formation of spurious by-products during the amplification process (Muyzer *et al.,* 1993). Primer extension was carried out at 68°C for 5 min. The PCR products were analyzed first by electrophoresis in 1% (wt/vol) agarose gel under ultraviolet (UV) illumination after ethidium bromide staining of the gel. Then, the amplified samples were separated by using DGGE (DCode System; Bio-Rad, Hercules, CA, USA) on 8% acrylamide gels with 30-60% formamide/urea gradients. Electrophoresis was performed at 60°C and 100 V over 18 h in 0.5 x TAE buffer (Tris-acetate, 0.04 M; EDTA, 0.001M). After that, the gels were rinsed in ddH2O, fixed in a solution of 10% ethanol, 0.5% acetic acid and silver stained. The DNA bands on the gel were visualized following electrophoresis by using a computer scanner (HP Scanjet G2410, UK).

A total of 34 different bands were gently excised from the gel. The gel and the bands were examined by manually observing some common bands to all dietary treatments, while those restricted to individual treatment were chosen for further analysis and sequencing. DNA of the gel plugs was amplified as described previously for pre–DGGE PCR and then subject to electrophoresis using a 2% agarose gel. Purified PCR products were then ligated into pGEM–T Easy vector (Promega, Madison, USA) and transformed into *E. coli* Top10 cells. Sequencing was performed on 34 transformed colonies representing the bands using the T7 primer found within the pGEM–T vector. A more definitive analysis of the population diversity was undertaken by comparing the 16S rRNA gene sequences of the isolates from this study with those found in public databases of NCBI.

Sequences were edited manually using CHROMAS Lite (ver. 2.0) before being assembled using the programme called SEQMAN (ver. 3.34, DNASTAR Inc.). The sequence data were aligned using Greengenes (DeSantis *et al.* 2006) and their nearest-neighbour for each sequence was identified. Both the chickens intestinal clones and their nearest neighbour sequences were manually imported into the ARB software package release 07.07.11 (Ludwig *et al.,* 2004) for fine alignment with similar sequences and to remove any alignment errors. A neighbour-joining tree was constructed (Saitou & Nei, 1987) for each enrichment media using the Kimura-2 parameter (Kimura, 1980) model in PHYLIP (Felsenstein, 1993) with 1000 bootstrap re-samplings.

Phylogenetic Analysis

Clone sequences generated from the two segments of intestinal tract were assigned to phylotypes that were designated by "SI/LI" which stands for small and large intestine followed by treatments given (T1/T2) and the numbers which represent the clone number. The 34 sequences from the current study were aligned against each other, while 14 additional sequences were identified as nearest-neighbours and downloaded from

the Greengenes website. The sulphate-reducing archaea, *Archaeoglobus fulgidus* strain VC-16 (Klenk *et al.,* 1997) was used as the out-group for the tree.

RESULTS AND DISCUSSION

In total, 34 clones were successfully sequenced, and of these clones, 11 clones originated from T1, and the remaining 23 clones originated from T2. The sequence analysis of the clones revealed 13 sequences (38.2%) with 99% identity to known bacteria, while 13 sequences (38.2%) had 95 to 98% identity to their nearest relatives. The remaining eight sequences (23.6%) had <95% identity to any recorded entries in Greengenes or GenBank. Of the 34 clones examined, the most abundant sequences were homologous to Lactobacillaceae (79.4%), while Clostridiaceae and Streptococcaceae each accounted for 8.8% and 5.8% of the total (see Fig.1). The remaining clones were represented by the families Bacteroidaceae and Enterococcaceae, with each accounted for 3.0%.

The diversity and composition of the microorganisms at different sites of intestinal tract are shown in Table 2. At the species level, *Lactobacillus acidophilus* was the dominant species present in the intestinal tract and this was represented by 38.2% of the 34 clones, followed by *Lactobacillus salivarius* with 23.5%, *Lactobacillus reuteri* (14.7%), and *Clostridium* sp. (8.8%). Apart from these, 5.8% of the clones were related to *Streptococcus bovis* and the remaining clones were related to *Bacteroides uniformis, Enterococcus cecorium* and *Lactobacillus johnsonii* (Fig.2). Salanitro *et al.* (1974) enumerated anaerobic bacteria from the small intestinal tract of 14-day-old chicks and showed that the predominant bacteria retrieved from the small intestine were largely dominated by *Lactobacillus* spp. (33.8 to 59%), followed by *Streptococcus* spp. (8.9 to 16.8%), *E. coli* (14.7 to 33%) and eubacteria (9 to 24.3%). Meanwhile, species-to-species comparison between the small and large intestinal tracts of the birds revealed that *Lactobacillus reuteri*

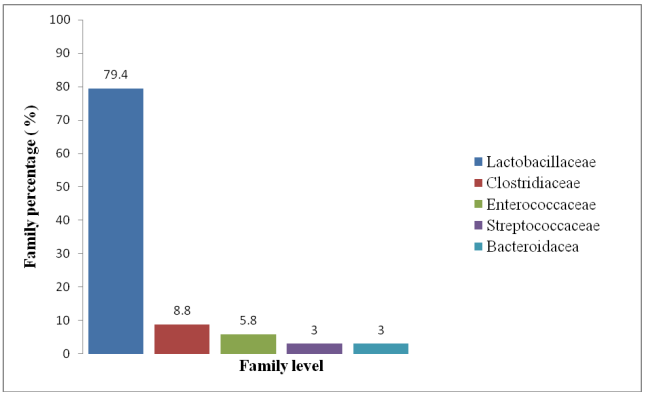

Fig.1: Distribution of bacteria derived from DGGE bands identified using the V3 region of 16S rDNA at family level

and *Lactobacillus salivarius* appeared to be present in both sections of the intestinal tract.

The topology of the phylogenetic tree is shown in Fig.3. Of the 34 clones, 33 clones were clustered together with the *Lactobacillaceae, Streptococcaceae, Enterococcaceae* and *Clostridiaceae* families, while one clone derived from the family Bacteroidaceae was not included in the tree because its distance of similarity is less than 90 %. In the current study, 27 clones grouped within the family *Lactobacillaceae* were closely affiliated with member of the genera *Lactobacillus,* and an uncultured bacteria clone from the ileum and cecum of chicken (Bjerrum *et al.,* 2006). Two clones [SI(T3)_104 and SI(T3)_105] were

TABLE 2
The origin of the different clones, their closest related microorganisms and % identity

Origin	Treatment	No. of clones [a]	Nearest related microorganism	% sequence similarity
Small intestine	T1/T2	13	*Lactobacillus acidophilus* NCFM	98
	T1/T2	7	*Lactobacillus salivarius* UCC118	95
	T1/T2	2	*Lactobacillus reuteri* 100-23 ctg2179	94
	T1	1	*Enterococcus cecorium*	97
	T2	1	*Lactobacillus johnsonii* NCC 533	99
	T2	3	*Clostridium* sp. S6	90
	T2	2	*Streptococcus bovis* SB5	97
Large intestine	T1	3	*Lactobacillus reuteri* 100-23 ctg2179	100
	T2	1	*Bacteroides uniformis* ATCC 8492	88
	T2	1	*Lactobacillus salivarius* UCC118	95

[a] A total of 34 clones were examined

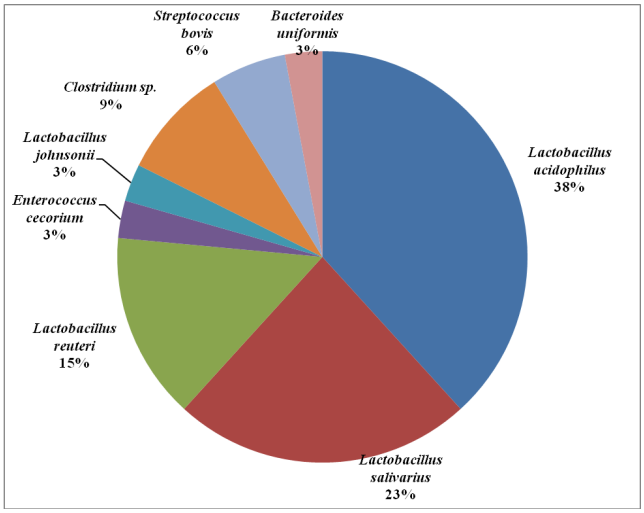

Fig.2: Distribution of bacteria derived from DGGE bands identified using the V3 region of 16S rDNA at species level

grouped within the Streptococcaceae and formed a cluster with *Streptococcus bovis*. Three cones were grouped together within the family *Enterococcaceae* and formed a cluster with *Clostridium* sp. Meanwhile, the remaining clone [SI(T1)_019] was grouped within *Enterococcus cecorum*, while clone LI (T2)_021 was grouped with an uncultured Turkey cecum clone (Scupham, 2007) (Fig.3).

The response of *Lactobacillus* spp. on xylanase supplementation in the diet appeared to have an effect, indicating that this group of bacteria is influenced by nutrient composition that was mediated by enzyme treatment (Teresa *et al.,* 2009). A study by Teresa and her co-workers (2009) on broilers fed wheat-soyabean-rape diets supplemented with xylanase preparation revealed that *Lactobacillus* spp. appeared

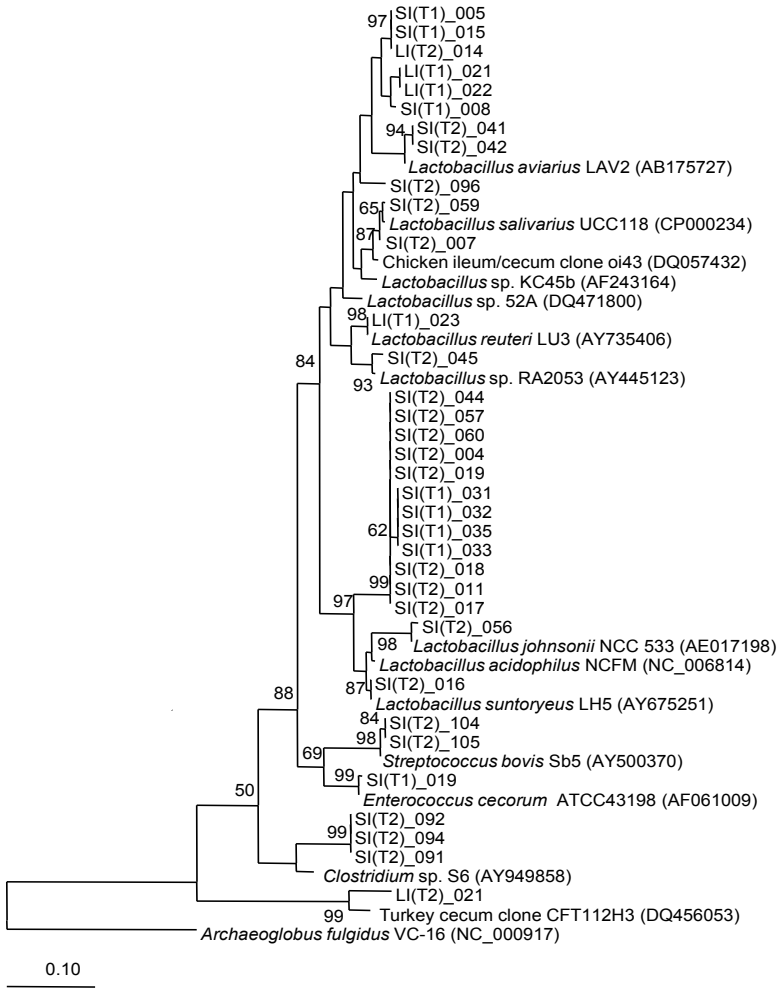

Fig.3: Distance-related phylogenetic tree of the lactic acid-producing bacteria from the different segments of the chicken intestine. Bootstrap values less than 50 are not indicated. The scale bar represents 10% sequence divergence. *Archaeoglobus fulgidus* VC-16 sequence was used as an out-group for rooting the tree.

to be present in high number. This finding is in agreement with the result of the present study, where high numbers of clone closely related with *Lactobacillus* spp. (79.4%) were detected from the intestine of the poultry fed with basal diet supplemented with xylanase. *Lactobacillus* spp. is considered as one of the most important bacterial groups that maintains the equilibrium of the microbial ecosystem (Pryde *et al.,* 1999). Our findings are similar, with *Lactobacillus* and *Streptococcus* accounting for 79.4% and 5.8% of the microflora, respectively. Various species of *Lactobacillus* have been extensively used as feed additives, either as mixed or pure cultures, and are considered beneficial to the monogastric animals (Fuller, 1999). The most dominating bands in this study were common for all the treatments and these belonged to *Lactobacillus acidophilus* and *Lactobacillus salivarius*.

High numbers of clone closely related with *L. acidophilus* were detected from the intestine. In broiler nutrition, this species, together with many other *Lactobacilli*, has been used as one of potential candidates for probiotics and has a beneficial effect on broiler performance (Kalavathy *et al.,* 2003). Supplementation of *L. acidophilus* cultures to chickens has significantly increased (P<0.05) the levels of amylase after 40 d of feeding trials (Jin *et al.,* 2000). It is believed that the lactobacilli colonizing the intestine may secrete the enzyme, therefore increasing the intestinal amylase activity (Sisson, 1989). The clones recognized as *L. salivarius* were detected in both the small and large intestinal tracts regardless of the treatment given. This is in agreement with that of a study by Al-Jassim *et al.* (2005) on the genetic diversity of lactic acid producing bacteria in the equine gastrointestinal tract, where *L. salivarius* were detected from the intestinal tract.

It is important to note that the PCR-DGGE approach allows determination of bacterial populations comprising more than 9% of the intestinal bacterial community (Zoetendal *et al.,* 1998). As it was not possible to obtain sequences from all bands in the gels and most of the retrieved sequences could not be aligned to any known bacterial species, it requires further investigation, particularly in relation to which intestinal microbiota could benefit from the xylanase supplementation and other methods such as the construction of 16S rRNA gene clone library that may be more suitable for this purpose.

CONCLUSION

Based on the findings of the current study, *Lactobacillus* spp. appeared to be the predominant bacteria across the different segments of the gastrointestinal tract of chicken. Further analysis is carried out to investigate the diversity using different molecular tools.

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Antioxidant Properties of Two Varieties of Bitter Gourd (*Momordica charantia*) and the Effect of Blanching and Boiling on Them

Choo, W. S.*, Yap, J. Y. and Chan, S. Y.

School of Science, Monash University Sunway Campus, Jalan Lagoon Selatan, 46150 Bandar Sunway, Selangor, Malaysia

ABSTRACT

The antioxidant properties of whole fruits and fruits without seeds and pith of two varieties of *Momordica charantia* (commonly known as bitter gourd), var. minima and var. maxima were investigated. The antioxidant content was investigated using ascorbic acid and total phenolic contents whereas the antioxidant activity was investigated using 2,2-diphenyl-1-picryl hydrazyl radical scavenging activity and ferrous ion chelating activity. The results showed that the fruits without seeds and pith and whole fruits of the two varieties of *Momordica charantia* exhibited different antioxidant content and activities. The ascorbic acid content ranged from 8.12 mg/100g to 24.46 mg/100g whereas the total phenolic content ranged from 1.47 mg GAE/100g – 27.23 mg GAE/100g. The antiradical power ranged from 4.67 to 5.94 and the ferrous ion chelating activity using the fruit extract concentration of 0.34 g/mL ranged from 10.6% to 89.3%. The effect of blanching and boiling on the antioxidant properties of fruits without seeds and pith of *Momordica charantia* var. maxima and minima was also investigated. Blanching and boiling of fruits without seeds and pith of *M. charantia* var. maxima and var. minima induced changes in the antioxidant content differently. The radical scavenging activity of the fruits without the seeds and pith of *M. charantia* var. maxima and var. minima increased as a result of blanching and boiling but their ferrous ion chelating activity became undetectable. There was no clear correlation between the antioxidant content and the antioxidant activities.

Keywords: Bitter gourd, antioxidant, ascorbic acid, phenolic, radical scavenging activity

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E-mail addresses:
choo.wee.sim@monash.edu (Choo, W. S.),
jyyap3@student.monash.edu (Yap, J. Y.),
sycha46@student.monash.edu (Chan, S. Y.)
* Corresponding author

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INTRODUCTION

Momordica charantia L. Cucurbitaceae is commonly known as bitter gourd, bitter melon or balsam pear (Marr *et al.,* 2004; Krawinkel & Kedig 2006). It is a member of the cucurbit family commonly grown in tropical and subtropical countries. The cultivated *M. charantia* is divided into two groups, which are fruits with a diameter less than 5cm known as var. minima and fruits with a diameter more than 5cm in diameter known as var. maxima (Reyes, 1994). *M . charantia* is grown for its edible fruit in African, Asian and South American countries including the Caribbean (Basch *et al.,* 2003). The immature fruits of *M. charantia* can be prepared in several ways and the seeds and pith are usually discarded before cooking. Besides frying and cooking, they can be dehydrated, pickled or canned. Pre-treatment such as blanching or soaking in salt water is done to reduce the bitter taste. The fruits, flowers and young shoots can also be used as flavorings (Marr *et al.,* 2004; Morgan & Midmore, 2002). The whole fruit of *M. charantia* (including the seeds and pith) can be used to produce juice as a health drink. According to Morgan & Midmore (2002), the fruits are a good source of vitamin C and also provide vitamin A and B, phosphorus, calcium, potassium and iron. The mineral and vitamin concentration of *M. charantia* is superior to that of other members of Cucurbitaceae pear (Marr *et al.,* 2004). *M. charantia* possesses some medicinal properties such as anti-diabetic, anti-tumor (Budrat & Shotipruk, 2009; Fang & Ng, 2011), anticancer, anti-inflammatory, antiviral, cholesterol lowering effects (Budrat & Shotipruk, 2009). The antidiabetic properties of *M. charantia* are due to charantin, vicine, polypeptide-p and other bioactive components such as antioxidants (Krawinkel & Kedig 2006). *M. charantia* is used as a topical internal or external treatment of wounds for management of worms and parasites infection (Grover & Yadav, 2004; Wu & Ng, 2008).

Alteration in the total antioxidant content and activity due to different processing methods is of scientific importance as it has a direct impact on dietary nutrition. Blanching is a treatment of vegetables to inactivate enzymes such as polyphenol oxidase, catalase, peroxidase, lipogenase, and chlorophylase (Ahmad & Shivhare, 2006). Blanching is also used as surface disinfectants to destroy microorganisms and cleans the dirt of the vegetables. It makes vegetables more compact, bright in colors and also hinder the loss of vitamins (Zheng & Lu, 2011). On the other hand, boiling is a treatment that softens vegetables by breaking down the cell walls, so as to make it consumable (Yao & Ren, 2011).

The main objectives of this study were (i) to determine the ascorbic acid and total phenolic contents of whole fruits and fruits without seeds and pith of the two varieties of *M. charantia*, namely *M. charantia* var. maxima and var. minima, (ii) to evaluate their antioxidant capacity (free radical scavenging activity and ferrous ion chelating activity), and (iii) to examine the effects of thermal treatments (boiling and blanching) on the antioxidant properties of

the fruits without seeds and pith of the two varieties of *M. charantia*.

MATERIALS AND METHODS

Materials

Young, emerald green fruits of *M. charantia* var. minima and var. maxima were used in this study. The fruits were obtained from a local supermarket in Selangor, Malaysia. All chemicals and solvents were of reagent-grade level and purchased either from Sigma-Aldrich (U.S.A.) or Merck (Germany).

Sample Preparation

The sample preparation was carried out according to the method of Lim *et al.* (2007) with some modifications. Whole fruits and fruits without seeds and pith for the two varieties of *M. charantia* were prepared by washing the fruits and wiping them to dryness. Seventy grams of samples were cut and crushed to a paste-like state with 25 mL of water for 2 mins (with intermittent stops to minimize heating) using a Waring blender. The homogenized sample was transferred into a 250 mL volumetric flask and top up to the mark with 50% ethanol. The mixture was mixed for 15 mins and then filtered under suction. Centrifugation was carried out a 1500 × g to obtain a clear supernatant liquid, which was used for subsequent assays. The extracts were stored at -20^{o}C and all tests were performed within a week. All assays were carried out in triplicate from different fruit samples.

Thermal Treatment

Blanching and boiling of the fruits of the two varieties of *M. charantia* were carried out according to the modified methods of Yao & Ren (2011) and Myojin *et al.* (2008). Fruits of *M. charantia* were washed and wiped dry. The fruits were cut lengthwise to remove the seeds and pith, and sliced into 1 cm thickness. Thermal treatment of the fruits was carried out by immersing the fruit slices in boiling water for an assigned time period [blanching (4 mins); boiling (10 mins)] and drained for 1 min. Four minutes of blanching was needed to inactivate the peroxidase in the fruits. The fruit slices were then transferred to ice water bath (ratio of ice to water was 1:4) for 2 mins to halt the heating process and drained for 1 min. An uncooked sample with no treatment was used as a control . Sample extraction was then carried out according to the sample preparation method mentioned previously.

Ascorbic Acid Content

The ascorbic acid content was determined by the iodine titration method (Suntornsuk *et al.,* 2002) or the RP-HPLC method: Waters Symmetry C-18 column (3.9 × 100 mm, 5 µm particle size), mobile phase 5% acetic acid, flow-rate 0.5 mL/min and 254 nm detection wavelength. Both methods gave similar results to within 5%.

Total Phenolic Content

Total phenolic content was determined according to the method of Lim *et al.* (2007). Extract (0.3 mL) was placed into test tubes followed by the addition of 1.5 mL of Folin-

Ciocalteu's reagent (diluted 10 times with water) and 1.2 mL of sodium carbonate (7.5% w/v). The test tubes were covered with parafilm, vortexed and allowed to stand for 30 mins. The absorbance was measured at 765 nm against a reagent blank. If the sample absorbance exceeded 1, the sample was diluted appropriately to give reading less than 1. A standard calibration curve was prepared by using gallic acid. Total phenolic content was expressed as gallic acid equivalents (GAE) in mg per 100g of fruits. As ascorbic acid contributes to the formation of blue molybdenum-tungsten complex, absorbance originating from it was corrected by measuring an ascorbic acid calibration curve.

Free Radical Scavenging Activity using 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH)

The free radical scavenging activity was determined according to the method of Suja *et al.* (2005) with some modifications. Sample (1 mL), each with different concentrations was added into 2 mL of 0.02g/L DPPH solution in ethanol. Absorbance at 517 nm was taken after allowing the solution to stand for 30 mins. The amount of sample needed to decrease the initial DPPH concentration by 50% (EC_{50}) was calculated graphically. The anti-radical power of extract was calculated as the reciprocal of EC_{50}.

$$\% \text{ remaining radical} = \left(\frac{A_{sample}}{A_{control}} \right) \times 100$$

Ferrous Ion Chelating Assay

The ferrous ion chelating assay was determined according to the method of Lim *et al.* (2009). Two millimolar of iron sulphate and 5 mM of ferrozine were prepared and diluted for 20 times. Extract (1 mL), each with different concentrations was mixed with 1mL of diluted iron sulphate, followed by 1mL of diluted ferrozine. The tubes were mixed well and allowed to stand for 10 mins at room temperature. The ability of the sample to chelate ferrous ions was calculated and expressed as: Chelating effect (%) = $[(A_{control} - A_{sample}) / A_{control}] \times 100\%$

Statistical Analysis

Data were interpreted by one-way analysis of variance (ANOVA) with Duncan's multiple range test using SAS software package (SAS Institute Inc, Cary, NC, USA). The statistical significance was evaluated at $p<0.05$ level.

RESULTS AND DISCUSSION

Ascorbic Acid Content

The fruits without seeds and pith for the two varieties of *M. charantia* were higher in ascorbic acid contents as compared to those of whole fruits (Table 1). Since the same amount of *M. charantia* was used for the ascorbic acid analysis, this indicates that the ascorbic acid content in the flesh of *M. charantia* fruits was higher than that in the seeds and pith. The ascorbic acid contents of fruits without seeds and pith of *M. charantia* in this study were lower than those reported by Iqbal *et al.* (2006) [85mg/100g fruit] and Myojin *et al.* (2008) [79.7mg/100g

fruit] but were higher than those reported by Somsub *et al.* (2008) [3.8-8.8 mg/100g]. The variety of *M. charantia* fruits used by these authors, however, was not mentioned. This difference is most likely due to the strong influence by genotype differences and external factors such as environmental conditions, maturity stage, harvest and post-harvest practices. The ascorbic acid content of *M. charantia* fruit in this study was higher than those of banana (4.9 ± 0.6 mg/100g) and mangosteen (5.8 ± 0.8 mg/100g) but were lower than those of guava (144 ± 60 mg/100g), papaya (108 ± 16 mg/100g) and orange (67 ± 9 mg/100g) [Lim *et al.,* 2007]. Hence, fresh *M. charantia* fruits can be a source of ascorbic acid.

The blanched and boiled samples of *M. charantia* var. maxima and var. minima showed higher ascorbic acid content as compared to the uncooked samples (Table 2). This is most likely due to the inactivation of ascorbic acid oxidase by heat induced during blanching and boiling. Heating prior to matrix disruption retains ascorbic acid effectively, as heat will first inactivate enzymes, thus avoiding the situation where enzymes are being transferred to the other parts of the fruits during matrix disruption, particularly ascorbic acid oxidase which takes part in ascorbic acid degradation (Munyaka *et al.,* 2010). Boiled samples had significantly lower ascorbic acid content as compared to that of blanched samples (Table

TABLE 1
Ascorbic acid content of uncooked *M. charantia* var. maxima and var. minima

	Ascorbic acid content (mg/100g)
M. charantia var. minima, whole fruit	8.12 ± 0.14^{c}
M. charantia var. minima, fruit without seeds and pith	13.79 ± 2.22^{b}
M. charantia var. maxima, whole fruit	8.44 ± 1.56^{c}
M. charantia var. maxima, fruit without seeds and pith	24.46 ± 0.24^{a}

^{abc} Values with different superscript letter within a column indicate significant difference at $p<0.05$

TABLE 2
Ascorbic acid content, total phenolic content, EC_{50} and anti-radical power of fruits without seeds and pith of *M. charantia* var. maxima and var. minima after thermal treatment

	Ascorbic acid content (mg/100g)	Total phenolic content (mg GAE/100g)	EC_{50} (mg/mL)	Anti-radical power
M. charantia var. minima				
Uncooked	20.91 ± 0.80^{b}	9.33 ± 0.77^{bc}	0.43 ± 0.04^{a}	2.44 ± 0.16^{d}
Blanched	29.53 ± 1.87^{a}	10.42 ± 0.96^{b}	0.05 ± 0.01^{c}	20.93 ± 1.59^{b}
Boiled	23.33 ± 2.96^{b}	5.80 ± 2.30^{cd}	0.06 ± 0.01^{c}	18.23 ± 1.35^{c}
M. charantia var. maxima				
Uncooked	11.98 ± 1.48^{c}	14.74 ± 2.46^{a}	0.38 ± 0.04^{b}	2.63 ± 0.36^{d}
Blanched	27.11 ± 0.32^{a}	7.60 ± 2.53^{bcd}	0.04 ± 0.00^{c}	24.09 ± 1.35^{a}
Boiled	22.32 ± 0.63^{b}	5.16 ± 2.99^{d}	0.06 ± 0.00^{c}	17.35 ± 0.22^{c}

Values^{abcd} with different superscript letters within a column indicate significant difference at $p < 0.05$

2). This is in accordance with the study of Somsub *et al.* (2008) on the effect of boiling and blanching on the vitamin C content of *M. charantia* although the variety was not mentioned. Boling, being a more intense heat treatment than blanching, caused a greater degradation of ascorbic acid.

Total Phenolic Content

The total phenolic contents of uncooked, whole fruits of *M. charantia* were higher than those of fruits without seeds and pith (Table 3).). Since the same amount of *M. charantia* was used for the total phenolic content analysis, this indicates that the seeds and pith had a higher amount of phenolic compounds compared to the flesh of the fruit. The total phenolic contents of *M. charantia* fruits in this study were lower than those of Lin & Tang (2007) [143.6 ± 8.4mg GAE/100g] and Ng *et al.* (2011) [582.9 ± 47.9 mg GAE/100g]. Again, the variety of *M. charantia* fruits used by these authors was not mentioned. The difference in the total phenolic content was due to the different sample preparation method by these authors. Lin & Tang (2007) and Ng *et al*. (2011) determined the use of total phenolic content in powder form and lyophilized form, respectively, whereas liquid extract was used in this study. The total phenolic content of *M. charantia* fruit was comparable to those of dragon fruit (21 ± 6 mg/100g) and papaya (28 ± 6 mg/100g) but was lower than those of guava (138 ± 31 mg/100g), star fruit (131 ± 54 mg/100g) and orange (75 ± 10 mg/100g) [Lim *et al.,* 2007]. Blanching and boiling process showed different effects on the total phenolic content of the two varieties of *M. charantia* fruits (Table 2). Decreased phenolic content was found after blanching and boiling of the fruits without seeds and pith of *M. charantia* var. maxima. The decrease in phenolic compounds after blanching and boiling was in accordance with the studies of Myojin *et al.* (2008), Amin *et al.* (2006), Wen *et al.* (2010) and Miglo *et al.* (2008). These researchers reported that the phenolic compounds in the vegetables studied were sensitive to heat and the heat treatment caused a significant loss of phenolic content which leached into the water. There were no significant differences in the total phenolic content of fruits without seeds and pith of *M. charantia* var. minima after blanching and boiling compared to the uncooked samples (Table 2). This may be due to the phenolic compounds in *M. charantia* var. minima were more heat stable.

Free Radical Scavenging Activity

The concentration of a sample required to scavenge 50% of DPPH is termed as EC_{50}. The lower the $EC_{50,}$ the better it was able to scavenge the radicals. The increased amount of antioxidant in a given volume of fruit extract is responsible for the increased reduction of the DPPH solution (Lim *et al*., 2007). Phenolic compounds and ascorbic acid can act as free radical scavenger. Table 4 shows the EC_{50} and anti-radical power of *M. charantia* var. minima and maxima. The whole fruits of *M. charantia* var. minima had higher anti-radical power compared to the fruits without seeds and pith of the same

variety of *M. charantia*. With no significant differences in ascorbic acid contents (Table 1), this is most likely due to the much higher total phenolic contents in the whole fruits with the seeds and pith (Table 3) contributing significantly to the anti-radical power. Wu & Ng (2008) reported that the free radical scavenging activity of a wild variety of *M. charantia* fruit was contributed mainly by the presence of phenolic compounds. Kubola & Siriamornpun (2008) reported a positive correlation between the radical scavenging activity and phenolic content in water extract of leaf, stem and fruit fractions of *M. charantia.* However, the EC_{50} and antiradical power of whole fruits and fruits without seeds and pith of *M. charantia* var. maxima did not show any significant difference even though the total phenolic contents of whole fruits were significantly higher ($p>0.05$) than those of fruits without seeds and pith (Table 3). This may be due to its much lower ascorbic acid content (Table 1).

Table 2 shows that the anti-radical power of the blanched and boiled samples was higher than that of the uncooked samples. Comparing the total phenolic content in Table 2, the scavenging activity was not proportional to the phenolic compounds, which is not in accordance with the study of Myojin *et al.* (2008) and Ng *et al.* (2011) who reported a positive correlation between the radical scavenging activity and phenolic content of the blanched and boiled samples of *M. charantia,* respectively. On the other hand, comparing the ascorbic acid content with the anti-radical power in Table 2, it can be suggested that the radical scavenging activity of *M. charantia* in this study was mainly contributed by the ascorbic acids with minor contribution from the phenolic compounds. Ascorbic acid acts as a free radical scavenger and its reaction was faster

TABLE 3
Total phenolic content of uncooked *M. charantia* var. maxima and minima

	Total phenolic content (mg GAE/100g)
M. charantia var. minima, whole fruit	14.82 ± 5.27^{b}
M. charantia var. minima, fruit without seeds and pith	5.92 ± 1.40^{c}
M. charantia var. maxima, whole fruit	27.23 ± 1.87^{a}
M. charantia var. maxima, fruit without seeds and pith	1.47 ± 0.13^{d}

[abcd] Values with different superscript letter within a column indicate significant difference at $p<0.05$

TABLE 4
EC_{50} and anti-radical power of uncooked *M. charantia* var. minima and maxima

	EC_{50} (g/mL)	Anti-radical power
M. charantia var. minima, whole fruit	0.17 ± 0.03^{b}	5.94 ± 0.84^{a}
M. charantia var. minima, fruits without seeds and pith	0.21 ± 0.02^{a}	4.78 ± 0.36^{b}
M. charantia var. maxima, whole fruit	0.21 ± 0^{a}	4.67 ± 0.11^{b}
M. charantia var. maxima, fruits without seeds and pith	0.18 ± 0.02^{ab}	5.52 ± 0.70^{ab}

[ab] Values with different superscript letter within a column indicate significant difference at $p<0.05$

compared to other scavenging molecules such as polyphenols (Scalzo 2008; Zhang & Hamauzu, 2003). These results indicate that the contribution of phenolics and ascorbic acids to each sample's radical scavenging activity varied markedly from one to another, depending on their levels in each sample and/or synergistic effect of antioxidants.

Ferrous Ion Chelating Assay

Primary antioxidants scavenged radicals to inhibit chain initiation and break chain propagation whereas secondary antioxidants suppress the formation of radicals and protect against oxidative damage (Lim *et al.,* 2007). Ascorbic acid could act as primary or secondary antioxidant (Akoh & Min, 2008). *In vivo*, ascorbic acid donated hydrogen atoms as a primary antioxidant. Ascorbic acid was also capable of scavenging radicals directly by converting hydroperoxides into stable products. In foods, ascorbic acid act as a secondary antioxidant with multiple functions such as scavenge oxygen, shift the redox potential of food systems to the reducing range, acts synergistically with chelators and regenerate primary antioxidants (Bauernfeind & Pinkert, 1970). By forming a stable iron (II) chelate, an extract with high chelating power reduced the free ferrous ion concentration thus decreasing the extent of Fenton reaction which caused many diseases (Halliwell & Gutteridge, 1990). The chelating activity of whole fruits and fruits without seeds and pith for *M. charantia* var. maxima and var. minima is shown in Fig.1. It was observed that a gradual increment of chelating activity as the concentration of the fruit extract increased. The highest % chelating activity of whole fruits and fruits without seeds and pith of *M. charantia* var. maxima (0.34 g/mL) was 86.8% and 84.8%, respectively. The highest % chelating activity of whole fruits and fruits without seeds and pith of *M. charantia* var. minima (0.34 g/mL) was 89.3% and 10.6%, respectively. No study has been reported on the ferrous ion chelating activity on *M. charantia*, thus no comparison can be made. According to Lim *et al.* (2009), ferrous ion chelating activity did not correlate with the total phenolic content. In this study, ferrous ion chelating activity did not correlate with total phenolic content of whole fruits of the two varieties of *M. charantia.* It seems that ascorbic acids also do not contribute much to the ferrous ion chelating activity of fruits without seeds and pith of both varieties of *M. charantia*. The ferrous ion chelating activity was most likely due to chelating ligands in the fruits (Lim *et al.,* 2009). There was no ferrous ion chelating activity detected in the blanched and boiled samples (0.20 g/mL) [Table 5] although ascorbic acids and phenolic compounds were still detected in these samples (Table 2).

CONCLUSION

The results obtained in this study demonstrated that the whole fruits and fruits without seeds and pith of two varieties of *M. charantia* showed different levels of ascorbic acid (8.12 mg/100g – 24.46 mg/100g) and phenolic compounds (1.47 mg GAE/100g –

27.23 mg GAE/100g). Different magnitude of the free radical scavenging activity (primary antioxidant activity) and ferrous ion chelating activity (secondary antioxidant activity) of the two varieties of *M. charantia* was also demonstrated. The antiradical power ranged from 4.67 to 5.94 and the ferrous ion chelating activity using the fruit extract concentration of 0.34 g/mL ranged from 10.6% to 89.3%. The fruits of *M. charantia* can be made into juice as a drink with primary and secondary antioxidant activities. The primary antioxidant activity of the fruits without the seeds and pith of *M. charantia* var. maxima and var. minima increased as a result of blanching and boiling but their secondary antioxidant activity became undetectable.

TABLE 5
Ferrous ion chelating (FIC) activity (%) of fruits without seeds and pith of *M. charantia* var. maxima and var. minima (0.20 g/mL) after thermal treatment

	FIC activity (%)	
	Maxima	Minima
Uncooked	44.47 ± 0.56	73.59 ± 3.92
Blanched	Not detected	Not detected
Boiled	Not detected	Not detected

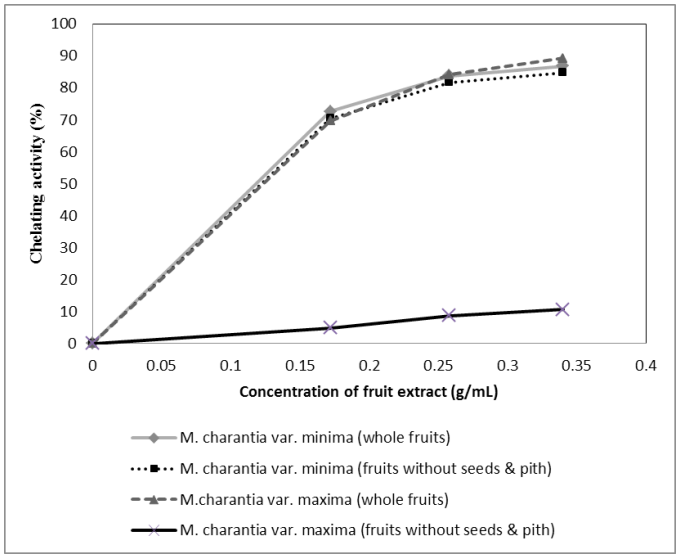

Fig.1: Ferrous ion chelating activity (%) of uncooked *M. charantia* var. maxima and minima

Note: The concentration of fruit extract was calculated by taking the weight of sample and divided it with the final volume of extract obtained.

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Induction of Skin Ulcers in Moon Light Gourami (*Trichogaster microlepis*) with *Aphanomyces invadans* Zoospores

Afzali, S. F., Hassan, M. D.* and Mutalib A. R.

Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Epizootic ulcerative syndrome (EUS) is one of the seasonal and economically devastating diseases in the wild and farmed fresh water and estuarine fish. Thus, an experimental study was conducted by the Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM) from February to April 2012, to examine the susceptibility of Malaysia's indigenous fish to EUS infection. In this experiment, forty apparently healthy moonlight gourami (*Trichogaster microlepis*) (10 ± 2 g body weight and 7.5 ± 1 cm in body length) were kept at 20 °C and challenged by intramuscular injection of zoospores (0.1 ml of 10,000 spores ml^{-1} suspension) of *Aphanomyces invadans* (isolate NJM9701). Fish were observed daily for characteristic EUS clinical signs during the 14-day trial and sampled at 1, 2, 4, 6, 7, 8, 9, 10, 11, 13, 12, 14 days post-injection. The infected skin and muscle were then sampled for histopathological examination. The results demonstrated that injected fish started to develop lesions that were histopathologically and grossly identical to those found in naturally EUS-infected fish and they died within two weeks after the infection. The profoundly penetrating ulcers had characteristics such as severe dermatitis, myofibrillar degeneration, and deep necrotizing granulomatous myositis. Therefore, the result of this study proved that moonlight gourami was vulnerable to the EUS agent.

Keywords: Epizootic ulcerative syndrome (EUS), Gourami, *Aphanomyces invadans*, Infection, Histopathology

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E-mail address:
hassanmd@upm.edu.my (Hassan, M. D.)
* Corresponding author

INTRODUCTION

Epizootic ulcerative syndrome (EUS) is an economically destructive disease that destroys a broad range of wild and farmed freshwater and estuarine fish particularly in Asia, Australia, some parts of USA and recently affected Africa as well (OIE 2009). Lilley and Roberts (1997) confirmed that

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the oomycete, *Aphanomyces invadans*, also known as *A. piscicida* was the causative agent of EUS (Lilley and Roberts 1997); where else Chinabut (1998) stated that the clinical signs of EUS include petechial hemorrhages, profound necrotic ulcers of the skin and reformed in swimming behaviors. Microscopically the lesions were distinguished by signs such as mycotic granulomas associated with penetrating hyphae of *A. invadans* and cellular feedback amongst muscles and fibers (Noga *et al.* 1988). EUS diagnosis's procedure is based on clinical signs or demonstration of the EUS-characteristic mycotic granulomas in histopathological test and molecular diagnostic methods namely, PCR (OIE 2006).

Bruno and Wood (1999) stated that highly vulnerable fish to EUS were *Channa* spp. and significantly *Channa striata*, barbs, major carps, gourami and mullets. So far, there hasn't been any in-depth research conducted in Malaysia regarding to *A. invadans* pathogenicity to Malaysian indigenous freshwater fish species despite the fact that Malaysia has been through severe EUS outbreaks in the 1990's. In the present study research, an anabantid fish i.e. Moonlight gourami (*T.microlepis*) was selected for the experimental infection due to its importance in the Malaysian aquarium industry and its popularity in Malaysia's market at affordable prices. Moonlight gourami belongs to the suborder Anabantoidei with over 100 various colored species that are being traded in the tropical fish industry currently. This would include about the numerous species of the genus *Trichogaster* containing many of the more popular gouramis traded in the industry (Cole *et al.* 1999).

This study is concentrated on moonlight gouramis to determine its vulnerability to the EUS disease agent for import risk analysis in the future because this fish is being widely exported to the other countries in the recent years; therefore it is crucial to obtain a comprehensive knowledge regarding the risks of disease introduction via international trades. The significance of this task is based on reducing the potential risk of pathogen's penetration into Malaysia's wild and farmed fish populations and also controlling and programming preventive measures in this regards.

MATERIALS AND METHODS

Oomycete Culture and Sporulation

Zoospores for this study were obtained from *A.invadans* fungal isolate NJM9701 (courtesy of Dr. Oitdmann, U.K.), cultured on GP-Penstrep agar (glucose-peptone agar added with penicillin-streptomycin) (OIE 2007). The modified method as described by Johnson *et al*. (2004) was utilized for zoospores production. *A. invadans* mycelia were allowed to grow on sterile hemp seeds planted on the GP agar. Later 6-mm growing hyphae on the hemp seeds were transferred into GPY broth (glucose-peptone-yeast broth added with penicillin-streptomycin), incubated at 25°C for five days and eventually washed three times in autoclaved pond water (APW).

The mycelium mats from tip of colonies with seeds were loaded into 1.5 ml micro tubes containing APW for 24 hours. The number of zoospores in the suspension was quantified by using a Neubauer counting chamber and adjusted to the required concentration. Briefly, an aliquot of culture was preserved in 10% neutral-buffered formalin (1 ml culture: 5 ml formalin), centrifuged for 10 min at 150 rcf. The pellet obtained was resuspended in 1.8 mL APW and a 10 µL aliquot was quantified with the Neubauer (Johnson *et al*. 2004).

Challenge experiments and histopathology

Fish used in this challenge experiments were local Moonlight gourami (*T. microlepis*) weighing an average of 10 ± 2 g body weight and 7.5 ± 1 cm in total body length, which were purchased from a local fish wholesale trader. 60 apparently healthy fish were acclimatized in 75-liter aquarium equipped with air stones and aquarium heaters and maintained at 20℃, for one month before the experiment to observe for any disease signs. Two replicate challenge groups and one control w ere set-up utilizing 20 fish per tank. Fish were anaesthetized using MS-222 (tricane methanesulphonate) prior to injection with 0.1 ml of a 10,000 spores ml^{-1} suspension (i.e. 1000 spores/fish) intramuscularly into the left side of the body below the dorsal fin using a 27-gauge needle. The control fish were inoculated with an equal volume of APW and all fish were observed daily for characteristic EUS clinical signs during the 14-day trial. Two fish were sampled at 1, 2, 4, 6, 7, 8, 9, 10, 11, 13, 12, 14 days post-injection (pi) and killed with an overdose of MS-222.The infected tissues were sampled for histopathological examination. The excised tissues were fixed in 10% phosphate buffered formalin solution for at least 24 hours, processed through automatic tissue processor and embedded in paraffin. The paraffin blocks were then sectioned at 4-5 µm thickness with a rotary microtome and mounted on clean glass slides. Slides were then stained with routine Hematoxylin and Eosin (H&E) (Prophet 1992), before mounted with coverslip prior for viewing under light microscope.

RESULTS AND DISCUSSION

Within 24 hours after injection, zoospore-injected and control fish both showed reddening and scale loss at the injection site which seemed to be due to traumatic injury caused by needle penetration. From day 4 to 7 pi, cotton-like, whitish colonies were observed at the site of inoculation just in zoospore-injected fish, and the presence of fungal mycelium was confirmed by wet mount preparation which showed thin aseptate branching hyphae (Fig.1a, Fig.1b).

Changes in swimming behavior, manifested by forward and backward teetering movements, started to happen in injected group from day 7 pi, while control fish appeared normal (Fig.2). Deep penetrating focal ulcers that exposed the underlying musculature started to appear from day 5 pi (Fig.3). Histopathology results showed characteristic EUS mycotic granulomas in injected fish from day 8 pi. Extensive mycotic and myonecrotic lesions

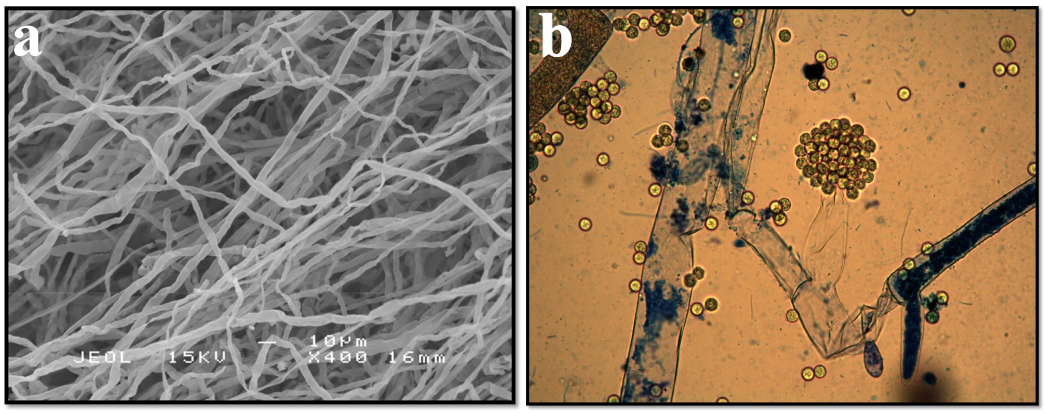

Fig.1: Showing (a) Electron microscopy of *Aphanomyces invadans* reisolated from injected gourami tissues showing non-septate hyphae. SEM, X400. (b) Light microscopy of *Aphanomyces invadans* reisolated from injected gourami tissues showing primary zoospores (65µm).

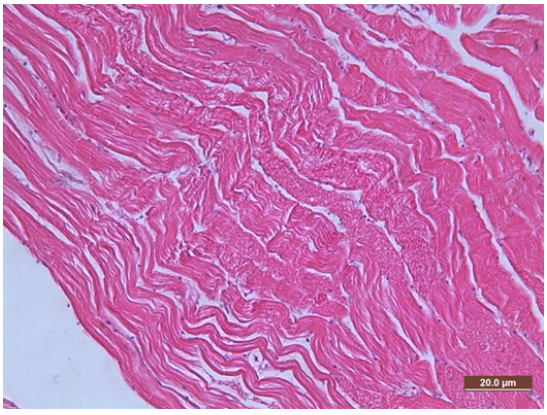

Fig.2: Longitudinal muscle section of APW injected gourami sampled at 13 days post injection. No microscopic lesion was observed as those seen in zoospore injected fish (H&E, X200).

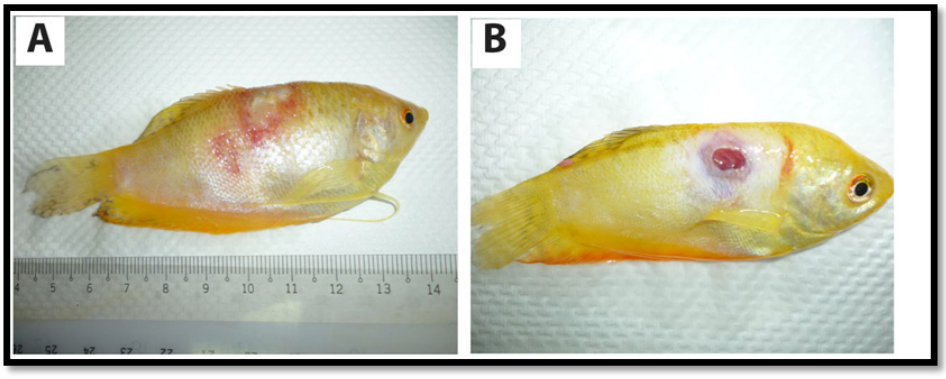

Fig.3: Experimentally infected gourami with *A. invadans* NJM 9701 at 100 spores/ml showed (A) necrotic ulcer surrounded with hyperaemic area, at day 6 post injection and (B) deep necrotizing ulcer at injection site seen at day 13, bordered with swollen hyperaemic ulcer and blanched margin.

were observed in muscles from day 6 pi. The injected muscle tissue area showed degenerating myofibrils infiltrated with inflammatory cells from day 8 (Fig. 4). Hyphae appearance could be detected in dermal and muscular layers. Large vacuoles were formed in degenerated muscles and granulomatous reactions were observed from day 9 pi (Fig. 5). Mortalities started to occur in injected fish from day 11 pi and by day 14 all injected fish were dead. All the dead fish had severe swollen hemorrhagic lesions with massive proliferation of hyphae in the lesion area of injected site similar to those caused by *A. invadans* in natural EUS infected fish.

This is the first research conducted in Malaysia on experimental infection of *Aphanomyces spp.* as an EUS aetiologic agent in Malaysian freshwater aquarium fish.

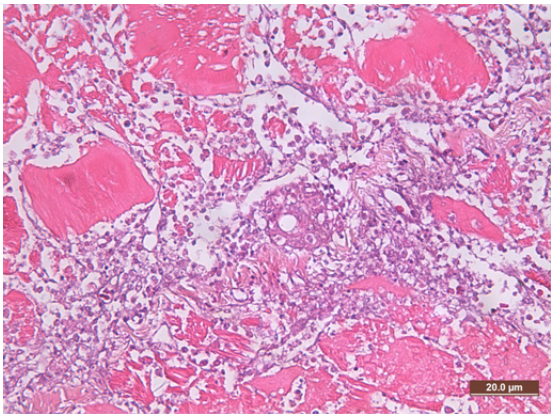

Fig.4: Histopathological section of zoospores-injected gourami sampled at 8 days post injection, showing degeneration of muscles fibres, interspersed with severe necrotic area and infiltrated with mononucleated inflammatory cells in lacunae-like spaces . Also seen mycotic granulomas (arrow).in the necrotic areas. Note the loss of muscles fibre architecture (H&E, X200).

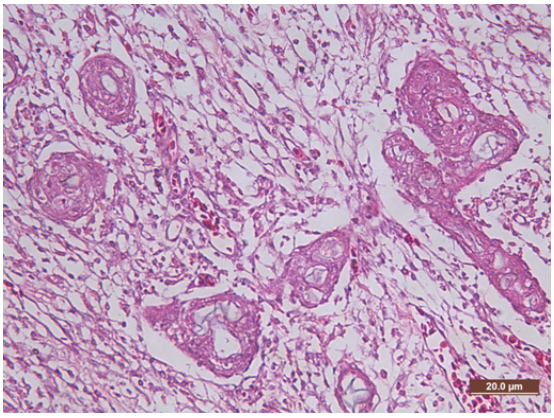

Fig.5: Histopathological characteristics of zoospores injected gourami sampled at 13 days post injection. Note the presence of mycotic granulomas (arrows), interstitial oedema and inflammatory cells infiltration in epidermis and dermis (H&E, X200).

This current study had successfully induced dermal ulceration in Moonlight gourami similar to natural EUS condition, via intramuscular injection of *A. invadans* strain NJM9701 live zoospores. Current findings indicated that the injected zoospores of *A. invadans* were able to grow in the muscles of Moonlight gourami. The zoospores were able to germinate into hyphae and proliferate extensively causing necrotizing granulomatous dermatitis with inflammatory response within very short period after injection. Lilley *et al*. (1998) stated that even though more than 100 fish species were reported to be affected by EUS, only a few of them had been validated by demonstrating the presence of mycotic granulomas in histological sections or by isolation of the pathogenic *Aphanomyces* fungus from tissues underlying the ulcers. Catap and Munday (2002) had also demonstrated EUS lesions in gourami (*T. trichopterus*), and sand whiting (*Sillago ciliate*) with injection of 0.05 ml of 1.7 ×1000 zoospores ml^{-1} suspension (85 spores/fish or 22 spores/g body weight. Both species of fish exhibited chronic granulomatous response and inflammatory cells, predominantly macrophages and lymphocytes, infiltrating the muscle and skin tissues at day 6–8 post-inoculation. This number of spores seems to be so much less in comparison with to our experiment but the research conducted by Khan *et al*. (1998) also reported dose regimes which varied by up to 7000–10,000 spores injected in various fish species and still obtained acceptable data. Mycotic granolumas were also observed in artificially infected ayu and carp (Wada *et al*. 1996). Experimental infection done in USA with *A. invadans* which were carried out in four species of estuarine fish lead to similar lesions, and severe pathologies in Atlantic menhaden (*Brevoortia tyrannus*) and Killifish were seen (Johnson *et al*. 2004). These results supported the results of current study which proved that some species of fish were sensitive than others, and fish having soft epidermal skin layers such as gourami was more vulnerable to be affected by *A. invadans.*

A limited number of researches have been conducted in utilizing bath challenge to expose fish to *A. invadans* spores. Atlantic menhaden which is one of the most vulnerable species to EUS was tested by aqueous exposure and confirmed that *A. invadans* was pathogenic via this route and also demonstrated that skin damage prior to pathogen exposure increased the prevalence of fish with ulcerous lesions and mortality (Kiryu *et al*. 2002; 2003). In addition, Sosa *et al*. (2007) demonstrated that more than 80% mortalities occurred in striped mullets (*Mugil cephalus*) injected with five spores per fish.

Oidtmann *et al*. (2008) had also conducted an experimental infection using similar *A. invadans* strain NJM9701 and showed that European catfish (*Silurus glanis*) produced typical EUS ulcerative skin lesions. In their study also, gouramis were used as positive control and showed to be more vulnerable to *A. invadans* infection as compared to European catfish. On the other hand, injected gouramies were indicated

changing in swimming behavior (forward and backward teetering movements) on Day 6 p.i similar to those were seen in moon light gourami on day 7 pi in present study. Saylor *et al*. (2010) described a mass mortality event of snakehead *Channa marulius* collected from freshwater bodies in Florida which clinical signs appeared within the first 2 days of captivity included petechiae, ulceration, erratic swimming, and inappetence which are similar to those are observed in this study.

Pradhan *et al.* (2008) discovered that, Indian major carp (*Catla catla)* greater than 1 year old seem to be resistant to infection by *A. invadans*, however, histopathological examinations were done by Baruah *et al*. (2012) revealed hyphae and granulomatous reactions in muscle tissue sections of Catla similar to those described in present study.

Since our knowledge on the vulnerability of fish species to EUS is still very limited, future studies should investigate the susceptibility of other local species especially those most likely to be affected in their natural environment. This will assist in assessing the most probable impact of an introduction of the pathogen into Malaysia and also provide useful information for import risk analysis of moonlight gourami to the other countries.

CONCLUSION

In conclusion, we have shown that moonlight gourami is vulnerable to an EUS pathogen, *A. invadans* via intramuscular injection. Therefore, it is recommended that EUS vulnerable species should not be cultured in high potential outbreak areas to avoid huge economic losses. However, since gourami is highly vulnerable to infection via inoculation; these species are ideal to be utilized as a laboratory model for further studies with *A. invadans* in Malaysia .

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Isolation and Characterization of *Lactobacillus* Strains as Potential Probiotics for Chickens

Shokryazdan, P.[1], Kalavathy, R.[2], Sieo, C. C.[1,3], Alitheen, N. B.[3], Liang, J. B.[4], Jahromi, M. F.[4] and Ho, Y. W.[1]*

[1]*Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[2]*Faculty of Pharmacy, Universiti Teknologi MARA, 40450 Shah Alam,Selangor, Malaysia*
[3]*Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*
[4]*Institute of Tropical Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia*

ABSTRACT

The ban on the use of antibiotics as growth promoters for poultry production in many countries has led to increasing interest to use probiotics as an alternative. In the present study, some *Lactobacillus* strains were isolated from chicken intestines, identified and assessed (*in vitro*) for their ability to survive and colonize the gastrointestinal tract (GIT), with a view to select suitable strains as potential probiotic candidates for chickens. Out of 42 isolated strains, three isolates, identified as *Lactobacillus* strains based on initial identification and tolerant to acid and bile based on preliminary screening using turbidity (optical density) as a measurement of growth, were selected for detailed identification and further *in vitro* assays. The three isolates were identified to species level using carbohydrate fermentation profile analysis and 16S rRNA gene sequencing. Results showed that all three strains belonged to *Lactobacillus salivarius*. The three *L. salivarius* strains were then assessed for their ability to tolerate the stress conditions in the GIT and capacity to adhere to the intestinal epithelial cells using *in vitro* assays of acid, bile and pancreatic enzyme tolerance measured by viable colony counts, and adhesion assay using Caco-2 cell line. The results showed that all three *L. salivarius* strains exhibited good tolerance to acid, bile and pancreatic enzymes and a strong ability to adhere to intestinal epithelial cells. Thus, they would be able to survive the stress conditions of GIT, as well as to attach and colonize the GIT, and could be considered as good potential candidates for probiotics of chickens.

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E-mail addresses:
parisa_shokryazdan@yahoo.com (Shokryazdan, P.),
kalav922@puncakalam.uitm.edu.my (Kalavathy, R.),
cssieo@upm.edu.my (Sieo, C. C.),
noorjahan@upm.edu.my (Alitheen, N. B.),
jbliang@upm.edu.my (Liang, J. B.),
mfjahromi@yahoo.com (Jahromi, M. F.),
ywho@upm.edu.my (Ho, Y. W.)
* Corresponding author

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Keywords: Chicken probiotic, *Lactobacillus* strains, *Lactobacillus salivarius*, probiotic *Lactobacillus*

INTRODUCTION

The poultry industry is one of the most important livestock industries in many countries, including Malaysia, and it contributes substantially to the economies of the countries. Prevention and control of poultry diseases would be necessary in order to avoid large economic losses. For over fifty years, antibiotics were routinely used to prevent or control diseases and to promote growth and feed efficiency (Kabir, 2009). However, with increasing concerns on the emergence of antibiotic resistant bacteria, the rampant use of antibiotics as a preventive tool for diseases and growth promotion was questioned (Patterson & Burkholder, 2003). The ban on subtherapeutic antibiotic usage for livestock production in Europe and the potential ban in the United States have led to an increasing interest in finding alternatives for antibiotics as growth promoters. Probiotics have been considered as one of the alternatives (Kabir, 2009). A probiotic is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). Every bacterial strain must have some special properties to be considered as a potential probiotic (FAO/WHO, 2001).

Lactobacilli are a group of bacteria that are frequently used as probiotics. They have a long history in their use as probiotics in the food industry and the *Lactobacillus* strains are "generally recognized as safe" (GRAS). They are commonly found in the environment such as soil, water, decaying plant materials, as well as in the normal microflora of the gastrointestinal tract (GIT) of animals (Kizerwetter-Swida & Binek, 2005). They have been used extensively in the food industry as starters in fermented products. In chickens, *Lactobacillus* strains are able to establish in the GIT within a day after hatching as they have a high ability to attach to the intestinal epithelial cells (Fuller, 1973). It has been suggested that in order to have more specific applications, bacterial strains intended as probiotics for chickens should be isolated from the natural microflora of the GIT of chickens (Kizerwetter-Swida & Binek, 2005).

As a result of the growing interest in probiotics, many purported probiotic products have been marketed without proper studies on the probiotic properties of the strains, giving rise to problems of inconsistent efficacy of the products. Several studies have reported misidentification or mislabelling of probiotic species or presence of unspecified species in many commercial probiotic products (Hamilton-Miller & Shah, 1996; Canganella *et al.,* 1997; Klein *et al.,* 1998; Hamilton-Miller *et al.,* 1999; Schillinger, 1999). In Malaysia, one of the reasons for the reluctance in the use of probiotics as an alternative to antibiotic growth promoters by poultry farmers is the inconsistency of the probiotic products' efficacy. Since the properties of probiotic are strain specific, the quality of products is closely linked to the individual strains in the products, thus, they should

be correctly identified and their probiotic properties properly studied. In 2001, FAO/WHO produced a set of guidelines for the evaluation of probiotics in food in which they recommended that every potential probiotic strain must be correctly identified, followed by various *in vitro* assays to investigate its functional properties and *in vivo* trials for its safety. This is because probiotic properties are strain specific and cannot be extrapolated to the whole genus or species. In the present study, some *Lactobacillus* strains were isolated from chicken intestines, identified and characterized for their probiotic properties, with a view to select suitable strains with probiotic attributes as potential probiotic candidates for chickens. The *Lactobacillus* strains were identified to species level using phenotypic and molecular characteristics and the primary probiotic properties (recommended by FAO/WHO, 2001) studied (*in vitro*) were the ones which would enable the strains to survive and colonize the GIT such as the abilities to tolerate acid, bile and pancreatic enzymes, and the capacity to adhere to the intestinal epithelial cells.

MATERIALS AND METHODS

Isolation of the Lactobacillus Strains

Samples of intestinal contents were collected from five healthy chickens at 42 days of age from the farm of the Department of Animal Science, Universiti Putra Malaysia. Chicken intestines were collected aseptically immediately after the chickens were euthanized. Intestinal tissues were washed with sterile phosphate buffer saline (PBS) (8g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 l distilled water, pH 7.2) to remove intestinal contents and surface mucus to obtain adhering bacteria. Intestinal epithelial tissues were scraped with a sterile blade to obtain 1 g of content. Then, 10-fold serial dilutions of up to 10^{-8} for each sample were prepared using sterile diluent (0.5% peptone in distilled water). From each dilution of 10^{-2} to 10^{-8}, 100 µl was streaked onto sterile petri dishes containing de Man, Rogosa and Sharpe (MRS) agar medium (Merck) and incubated for 72h at 37°C in anaerobic jars (Oxoid) containing gaspack (AnaeroGen, Oxoid). After incubation, well-formed colonies were randomly picked and streaked onto new MRS plates. The isolates were purified by subculturing them three times on MRS agar. Meanwhile, the stock cultures of pure isolates were stored in 20% glycerol at -80°C, and the cultures for studies were maintained routinely on MRS agar.

Initial Identification and Preliminary Screening of Isolates

For initial identification of the selected isolates, morphological examination, Gram staining and catalase test were performed. Overnight cultures of each isolate on MRS agar were used. The isolates were Gram stained and examined under a light microscope (Dialux, Leitz Wetzlar, Germany) for morphological characteristics. In the catalase test, 50 µl of 3% hydrogen peroxide was dropped on randomly selected colonies of each isolate on MRS agar. Effervescence from the colonies indicated

positive reaction. Only gram-positive and catalase-negative isolates (Kandler & Weiss, 1986; Schillinger & Lucke, 1987) were selected for a rapid preliminary screening of their acid and bile tolerance [growth measured as increase in turbidity determined by optical density (OD)] to select only those with good tolerance levels for detailed identification and further *in vitro* assays of their probiotic characteristics for survival in the stress conditions of GIT.

The preliminary screening for acid tolerance was according to Ehrmann *et al*. (2002) with modifications. Bacterial cells from overnight cultures in MRS broth (Merck) (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS and resuspended in sterile PBS at a final concentration of 7 to 8 log CFU/ml. The resuspended cells were then inoculated (1%, v/v) into PBS adjusted to pH 1, 2 and 3 with 1 N HCl (acidic condition) and normal PBS with pH 7.2 (control). After 3 h of incubation at 37˚C, 1% (v/v) of cell suspension was inoculated into 10 ml of fresh MRS broth and incubated at 37˚C for 24 h. After the incubation period, cell growth was assessed by measuring OD at 620 nm using a spectrophotometer (Barnstead International, USA). The isolates that showed at least 80% growth, in comparison with that of the control (100%), were selected as good acid tolerant strains. Three replicates were made for each isolate at each pH value. The experiment was carried out twice.

For the preliminary screening of bile tolerance, only the isolates that showed good acid tolerance were used. The bile tolerance test followed that of Jacobsen *et al.* (1999) with modifications. The overnight culture of each isolate (adjusted to a final concentration of 7 to 8 log CFU/ml) was inoculated (1%, v/v) into 10 ml of fresh MRS broth with or without (control) 0.3% oxgall (Sigma) and incubated at 37˚C for 4 h. After incubation, growth was assessed by measuring OD at 620 nm. The isolates that showed at least 80% growth, in comparison with that of the control (100%), were selected as good bile tolerant strains. Three replicates were made for each isolate and the experiment was carried out twice.

Identification of the Isolated Strains to Species Level

The isolates selected for their good acid and bile tolerance were identified to species level using a biochemical method [carbohydrate fermentation profile analysis by API system (Bio-Merieux)] and a molecular technique (comparative sequence analysis of the 16S rRNA gene). For identification using the API system, the overnight culture of each isolate on MRS agar was used and carbohydrate fermentation profiles of the isolates were investigated using API 50 CH kits according to the manufacturer's instructions. The strains were identified using API LAB Plus software version 3.3.2 (Bio-Merieux).

For 16S rRNA gene sequencing, the cells of overnight cultures of each isolate in MRS broth were harvested by centrifugation at 5000 x *g* for 10 min at room temperature and used for DNA extraction. The DNeasy Blood & Tissue Kit

(QIAGEN, Germany) was used to extract total DNA according to the manufacture's instructions. For amplification of the 16S rRNA gene, two universal primers, F27 (AGAGTTTGATCMTGGCTCAG) and R1492 (TACGGYTACCTTGTTACGACTT) were used (Lane, 1991; McDonald *et al.,* 1995), with the expected PCR product of 1.5 kb. The PCR amplification was performed in 50 µl reaction mixtures using a MyCycler Thermal Cycler (Bio-Rad, USA), as described by McDonald *et al.* (1995). The PCR conditions were initial denaturation at 94˚C for 4 min, 30 cycles of denaturation at 94˚C for 1 min each, annealing at 55˚C for 30 s, and at 72˚C for 2 min, and a final extension at 72˚C for 5 min. The PCR-amplified product was electrophorized (80 v, 50 min) on a 1% (w/v) horizontal agarose gel, followed by staining with ethidium bromide (2 µg/ml) for 10 min, and visualizing using an Alpha Imager Documentation and Analysis System (Alpha Innotech, USA). The PCR product with the expected size of 1.5 kb was excised and purified using MEGAquick-spin$^{(TM)}$ PCR & Agarose Gel DNA Extraction System (iNtRON Biotechnology, Korea). Then, each purified PCR product was cloned into *E.coli* plasmid using a TOPO TA cloning kit (Invitrogen, USA). Colonies containing the 16S rRNA gene inserts were screened, picked and cultured in Luria-Bertani broth. Plasmid extraction was performed using a DNA-Spin Plasmid DNA Extraction kit (iNtRON Biotechnology, Korea). DNA sequence analysis was carried out for plasmid with the unique insert using an ABI 373XL automated sequencer (Applied Biosystems, USA) at both directions to obtain the full sequence of the amplicons.

Sequence Alignments and Phylogenetic Inference

DNA sequence data sets were assembled using the Bioedit sequence alignment editor software, version 7.0.9.0 (Hall, 1999). Discrepancy nucleotides between forward and reverse sequences were edited based on their electropherograms. Similarity values were determined using the Basic Local Alignment Search Tool (BLAST) of the GenBank (NCBI). Sequences with ≥ 97% similarity to the previously published sequences were used as the criterion to indicate species identity (Stackebrandt & Goebel, 1994).

A phylogenetic tree was constructed based on the 16S rRNA gene sequence analysis, in which the analysis involved 25 nucleotide sequences consisting of 3 sequences of *Lactobacillus* strains obtained in this study, 21 sequences belonging to *Lactobacillus* species obtained from the GenBank and the sequence of *Lactococcus lactis* (AB 100803.1) which was used as the outgroup. Evolutionary analyses were conducted with MEGA5 software (Tamura *et al.,* 2011). The evolutionary history was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). Bootstrapping was performed for 1000 replicates and only bootstrap values (the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test) above 50%

were reported (Felsenstein, 1985). The evolutionary distances were computed using the Tamura 3-parameter method (Tamura, 1992). Only values above 70 to 80% are usually considered to indicate high support; in this study, the values above 50% were considered to avoid dispersion of species. Potential anormalous sequences of the 16S rRNA gene were examined by the Mallard (Ashelford *et al.,* 2005) and the Bellerophon (Huber *et al.,* 2004) programs. Nucleotide sequences determined in this study were deposited in the public database (GenBank, NCBI) using the Sequin program.

Characterization of Selected Lactobacillus Strains for Survival in the Gastrointestinal Tract

In vitro assays for acid, bile and pancreatic enzyme tolerance were employed to characterize the selected *Lactobacillus* strains for survival in the GIT. Although acid and bile tolerance of the strains had been carried out in the preliminary screening, growth was estimated as increase in turbidity, which was good for rapid screening of a large number of strains, but was not very accurate as it measured dead cells along with live cells. As acid and bile tolerance is a prerequisite for survival in the GIT, in the present *in vitro* assays, growth was measured as viable cells [colony forming units (CFU)] counts (CFU/ml), which is a more accurate measurement of growth. A probiotic *Lactobacillus* strain, *L. reuteri* C10, from a commercial multi-strain probiotic (StellarLac) for chickens, was used as a reference strain. This strain was kindly provided by Stellar Gen Ltd Co. The growth medium for all *Lactobacillus* strains was MRS agar or broth, and the cultures were incubated anaerobically at 37˚C.

***In vitro* assay for acid tolerance**

The acid tolerance assay followed that of Ehrmann *et al.*, (2002) with modifications. Bacterial cells from overnight cultures (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS (pH 7.2), then resuspended in sterile PBS adjusted to a final concentration of 7 to 8 log CFU/ml. The resuspended cells were inoculated (1%, v/v) into sterile PBS adjusted to pH 3 with 1 N HCl (acidic condition) and normal PBS with pH 7.2 (control), and incubated anaerobically for 3 h at 37˚C. After incubation, 10-fold serial dilutions (up to 10^{-7}) of each *Lactobacillus* strain were prepared using PBS. Then 100 µl of 10^{-4} to 10^{-7} dilutions from each sample was streaked on MRS agar plates and incubated anaerobically at 37˚C for 24 h. After incubation, viability of bacterial cells was assessed by colony counts (CFU/ml) on the plates (Jacobsen *et al.* 1999; Ehrmann *et al.* 2002; Paramithiotis *et al.* 2006; Bilige *et al.* 2009). Tolerance to acidic condition was estimated by comparing viable cell counts after exposure to acidic (pH 3) and normal (control) conditions. The assay was performed twice, each in triplicate.

***In vitro* assay for bile tolerance**

The bile tolerance assay was according to Jacobsen *et al.* (1999) with modifications. Overnight culture of each *Lactobacillus*

strain (adjusted to a final concentration of 7 to 8 log CFU/ml) was inoculated (1%, v/v) into 10 ml of fresh MRS broth with or without (control) 0.3% (w/v) oxgall (Sigma, USA) and incubated anaerobically at 37˚C for 4 h, after which 10-fold serial dilutions of up to 10^{-7} were prepared using PBS. Then 100 µl of 10^{-4} to 10^{-7} dilutions from each sample was streaked on MRS agar plates and incubated anaerobically at 37˚C for 24 h. After incubation, colonies on the plates were counted and enumerated as CFU/ml (Gilliland *et al.* 1984; Jacobsen *et al.* 1999; Paramithiotis *et al.* 2006). Bile tolerance was estimated by comparing viable cell counts in MRS with and without bile (oxgall). The assay was performed twice, each in triplicate.

In vitro assay for pancreatic enzyme tolerance

Tolerance to pancreatic enzymes was tested according to the method of Ronka *et al.* (2003) with modifications. Bacterial cells from overnight cultures (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS (pH 7.2), and resuspended in sterile PBS at a final concentration of 7 to 8 log CFU/ml. The resuspended cells were inoculated (1%, v/v) into 10 ml of the test solution [PBS containing 150 mM $NaHCO_3$ and 1.9 mg/ml pancreatin (Sigma); pH 8] and control solution (PBS, pH 7.2). The cultures were incubated anaerobically at 37˚C for 3 h. After incubation, 10-fold serial dilutions of up to 10^{-7} were prepared using PBS, and 100 µl of 10^{-4} to 10^{-7} dilutions from each sample was streaked on MRS agar plates. The plates were incubated anaerobically at 37˚C for 24 h, after which viability of bacterial cells was estimated by colony counts (CFU/ml). Tolerance to pancreatic enzymes was estimated by comparing viable cell counts of test solution and control solution. The assay was performed twice, each in triplicate.

Adhesion assay

The human intestinal epithelial cell line, Caco-2 cell line (ATCC 2102-CRL), purchased from the American Type Culture Collection (ATCC), was used in the adhesion assay. The Caco-2 cells were routinely grown to 80 to 85% confluent in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 20% (v/v) fetal bovine serum (FBS) (Sigma), 10000 IU/ml penicillin (Sigma) and 10 mg/ml streptomycin (Sigma). The procedure used for the adhesion assay followed that of Gopal *et al.* (2001) with modifications. A cell suspension (1×10^5 cell/ml DMEM) of Caco-2 cells was used for preparation of a monolayer of the cells on glass cover slips placed in six-well tissue culture plates. One ml of the cell suspension was added into each well of the plates containing fresh DMEM, and the plates were incubated overnight. Incubation for maintenance of cells and adhesion assay was at 37˚C in 5% CO_2. For each *Lactobacillus* strain, cells from overnight culture (10 ml) were harvested by centrifugation at 4000 × *g* for 10 min at 4˚C, washed three times with sterile PBS (pH 7.2), then resuspended

in sterile PBS buffer (pH 7.2) to a final concentration of 1×10^8 CFU/ml. Adherence assay was performed by adding 100 µl of bacterial suspension onto the washed (once with PBS) monolayer of Caco-2 cells in the well containing 2 ml of fresh DMEM and incubated for 1 h at 37˚C. After incubation, the monolayers were washed four times with PBS to remove unattached bacteria, fixed with 3 ml of methanol and incubated for 5 to 10 min at room temperature. The fixed monolayers were Gram stained and examined with a light microscope under oil immersion lens (Dialux, Leitz Wetzlar). Adherence was evaluated in 20 random microscopic fields and the number of adhered *Lactobacillus* cells per Caco-2 cell was determined (Jacobsen *et al.* 1999; Gopal *et al.* 2001; Ali *et al.* 2008; Pan *et al.* 2009). The assay was performed twice, each in triplicate.

Statistical analysis

Data of the *in vitro* assays for acid, bile and pancreatic enzyme tolerance were analyzed by one-way analysis of variance using the SAS (Statistical Analysis System, 2008) program version 9.2. Treatment means were compared using Duncan's new multiple range test, and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation, initial identification and preliminary screening

A total of 42 bacterial isolates displaying general morphological characteristics of *Lactobacillus* were isolated from chicken intestines. The cell morphologies of the 42 isolates, observed using light microscopy, showed that all 42 isolates were rod shaped, varying from short to long (1.1 to 5.7 µm long), and straight to crescent rods, arranged singly, in pairs or in short or long chains. Catalase test showed that of the 42 isolates, 37 isolates were catalase negative, and of these, 26 isolates were Gram positive. In the preliminary screening of acid tolerance, none of the 26 isolates survived at pH 1 and 2. Sahadeva *et al.* (2011) had also found that none of the tested probiotic strains in their study, which included *L. acidophilus*, *L. casei*, *L. casei* Shirota, *Streptococcus thermophilus* and *Bifidobacterium*, could survive for 3 h at pH 1.5. Earlier, Chan *et al.* (2005) demonstrated that even aciduric *Lactobacillus* strains such as *L. acidophilus* could not survive after 2 h of exposure to pH 2. The results of the preliminary screening showed that at pH 3, 14 of the 26 isolates showed 95.6 to 107.0% growth in comparison with that of the control (100%), which was considered as good acid tolerant isolates, and were then tested for bile tolerance. Of these 14 isolates, only three isolates showed 85.2 to 92.8% growth in the presence of bile when compared to that of the control (100%), and were considered as good bile tolerant isolates. From this preliminary screening, only three isolates, designated as CI1, CI2 and CI3, were selected for detailed identification and further investigation on their ability to survive the stress conditions of the GIT and to adhere to the intestinal epithelial cells.

Identification using API system and 16S rRNA gene sequencing

The three isolates were identified based on both phenotypic and genotypic characterizations because according to FAO/WHO (2001) guidelines, a combination of phenotypic and genotypic methods should be used for identification and speciation of probiotic strains. The results of the carbohydrate fermentation profiles of the three isolates are shown in Table 1. All three isolates were able to ferment galactose, glucose, fructose, maltose, mannitol, sorbitol, N-Acetyl-Glucosamine, lactose, melibiose, sucrose and raffinose. The results of identification of the isolates using carbohydrate fermentation profile analysis by the API system and the 16S rRNA gene sequencing are presented in Table 2. Based on the results of carbohydrate fermentation profile analysis by the API system, the three isolates were 98.8 to 99.9% similar to *Lactobacillus salivarius*. The results of the 16S rRNA gene sequencing also showed that all the three isolates were 99% similar to *L. salivarius* DQ444477.1 (from the GenBank) with 99% query coverage. Thus, there were no discrepancies in the identification of the three isolates using the carbohydrate fermentation profile analysis by the API system and using 16S rRNA gene sequencing. However, some studies have shown that the API carbohydrate fermentation profile analysis was not adequate for identification to species level for some *Lactobacillus* strains (Yin *et al.,* 2005; Khunajakar *et al.*, 2008). The 16S rRNA gene sequences of the three isolates (*L. salivarius* CI1, CI2 and CI3) were deposited in the GenBank database under the accession numbers JN188391 to JN188393 (Table 2).

TABLE 1
Carbohydrate fermentation pattern of the three isolated strains

No.	API CH50 Kit substrate	CI1	CI2	CI3
0	Control	–	–	–
1	Glycerol	–	–	–
2	Erythritol	–	–	–
3	D-Arabinose	–	–	–
4	L-Arabinose	–	–	–
5	Ribose	–	–	–
6	D-Xylose	–	–	–
7	L-Xylose	–	–	–
8	Adonitol	–	–	–
9	B-Methyl-D-Xyloside	–	–	–
10	Galactose	+	+	+
11	Glucose	+	+	+
12	Fructose	+	+	+
13	Mannose	+	+	+
14	Sorbose	–	–	–
15	Rhamnose	–	–	–
16	Dulcitol	–	–	–
17	Inositol	–	–	–
18	Mannitol	+	+	+
19	Sorbitol	+	+	+
20	A-Methyl-D-Mannoside	–	–	–
21	A-Methyl-D-Glucoside	–	–	–
22	N-Acetyl-Glucosamine	+	+	+
23	Amygdalin	–	–	–
24	Arbutin	–	–	–
25	Esculin	–	–	–
26	Salicin	–	–	–
27	Cellobiose	–	–	–
28	Maltose	–	–	–

TABLE 1 (*continue*)

No.	API CH50 Kit substrate	CI1	CI2	CI3
29	Lactose	+	+	+
30	Melibiose	+	+	+
31	Sucrose	+	+	+
32	Trehalose	+	_	_
33	Inulin	_	_	_
34	Melezitose	_	_	_
35	Raffinose	+	+	+
36	Starch	_	_	_
37	Glycogen	_	_	_
38	Xylitol	_	_	_
39	Gentiobiose	_	_	_
40	D-Turanose	_	_	_
41	D-Lyxose	_	_	_
42	D-Tagatose	_	_	_
43	D-Fucose	_	_	_
44	L-Fucose	_	_	_
45	D-Arabitol	_	_	_
46	L-Arabitol	_	_	_
47	Gluconate	_	_	_
48	2-Keto-Gluconate	_	_	_
49	5-Keto-Gluconate	_	_	_

+, positive reaction; –, negative reaction.

Fig.1 shows the phylogenetic tree based on the 16S rRNA gene sequence analysis, depicting the phylogenetic relationships among the three *Lactobacillus* strains and 21 *Lactobacillus* type strains obtained from the GenBank. *Lactococcus lactis* (AB100803.1) was used as the outgroup. Strains CI1, CI2 and CI3, isolated in this study, were clustered together and were monophyletic with *L. salivarius* DQ444477.1 with a bootstrap value of 100%.

Acid, Bile and Pancreatic Enzyme Tolerance

In the case of functional probiotic properties, the ability to tolerate the stress conditions of GIT is one of the most important criteria in the selection of a strain as a potential probiotic candidate (Ouwehand *et al.,* 1999). Among the stress conditions, the presence of acid, bile salts and pancreatic enzymes are the most important stresses which an orally taken probiotic would encounter in GIT. Thus, it is essential that a potential probiotic strain is able to tolerate these stressful conditions in order to survive in GIT.

In chickens, mucous glands only exist near the entrance of the crop, so the intraluminal pH of the crop is relatively basic compared to the pH value in the proventriculus and gizzard (Klasing, 1998), which ranges from 2.5 to 4.74 and food ingestion can take up to 1 to 3 h depending on feed size (Musikasang *et al.,* 2009). In many studies, pH 3 has been considered as a standard pH for investigation of acid

TABLE 2
Identification using API system and 16S rRNA gene sequencing

Isolate	Identification using API 50 CH		Identification using 16 S rRNA		
	Nearest matched species	Similarity (%)	Nearest matched species from GenBank	Similarity (%)	Accession number
CI1	*L. salivarius*	99.9%	*L. salivarius* (DQ444477.1)	99%	JN188391
CI2	*L. salivarius*	98.8%	*L. salivarius* (DQ444477.1)	99%	JN188392
CI3	*L. salivarius*	98.8%	*L. salivarius* (DQ444477.1)	99%	JN188393

tolerance of probiotic strains (Liong & Shah, 2005; Koll *et al.,* 2008; Sahadeva *et al.,* 2011). In view of these studies and the results of the preliminary screening of the isolated strains in the present acid tolerance assay, in which none of the strains was able to survive at pH 1 and 2, only pH 3 was used to investigate the acid tolerance of the three *L. salivarius* strains.

The results of the acid tolerance assay (Table 3) showed that all three *L. salivarius* strains could tolerate pH 3 for 3 h. However, *L. salivarius* CI1 and CI3, with 0.05 log units reduction in cell viability, exhibited

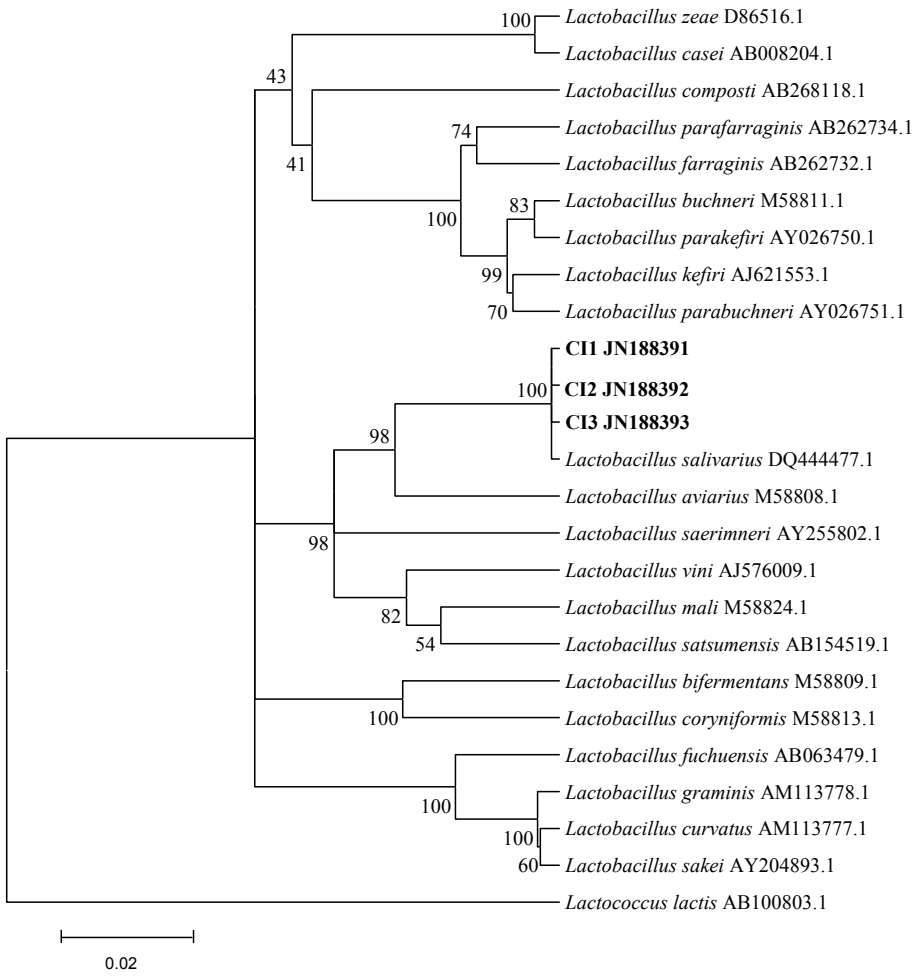

Fig.1: Phylogenetic tree based on 16S rRNA gene sequence analysis depicting the phylogenetic relationships among species of the genus *Lactobacillus* using the Neighbor-Joining method (Saitou and Nei, 1987). The outgroup was *Lactococcus lactis* (AB100803.1). The analysis involved 18 nucleotide sequences including three sequences of strains obtained in this study and 14 sequences belong to Lactobacillus species obtained from the GenBank (NCBI). Bootstrap values above 50% are indicated at the nodes of the tree. The scale bar represents 0.02-nucleotide substitutes per position.

significantly ($P < 0.05$) lower reduction in cell viability than *L. salivarius* CI2 and the reference strain *L. reuteri* C10, with 0.44 and 0.47 log units reduction in cell viability, respectively, which indicated a higher tolerance of *L. salivarius* CI1 and CI3 to the acidic condition than *L. salivarius* CI2 and the reference strain *L. reuteri* C10. Ehrmann *et al.* (2002) also found the strains of *L. reuteri*, *L. salivarius* and *L. animalis* to be tolerant to pH 3 for 4 h. Earlier, Charteris *et al.* (1998) reported in their review that most *Lactobacillus* spp were able to tolerate pH 4 for 1 h but the percentage of cell viability varied considerably among different strains.

In the chicken intestine, the total bile salt concentrations in the duodenum and cecum have been reported to be 0.175 and 0.008%, respectively (Lin *et al.,* 2003). In many studies, however, the standard level of 0.3% bile was considered for investigation of bile tolerance of potential probiotic *Lactobacillus* strains (Gilliland *et al.,* 1984; Jacobsen *et al.,* 1999; Boonkumklao *et al.,* 2006; Koll *et al.,* 2008; Ruiz-Moyano *et al.,* 2008; Sahadeva *et al.,* 2011). Thus, in the present study, 0.3% bile concentration was used. The results of the bile tolerance assay (Table 4) showed that all three *L. salivarius* strains exhibited bile tolerance (reduction in cell viability of 1.43 to 1.69 log units) at this concentration of bile salt, however, their tolerance levels were lower ($P < 0.05$) than that of the reference strain *L. reuteri* C10 (reduction in cell viability of 0.46 log units). A similar finding was reported by Koll *et al.* (2008) who found that all 67 *Lactobacillus* strains tested for their bile tolerance at 0.3% bile exhibited tolerance. Jin *et al.* (1998) also found that all 12 *Lactobacillus* strains studied were able to tolerate 0.3% of bile salt, while Jacobsen *et al.* (1999) reported that 41 of 42 tested *Lactobacillus* strains could tolerate bile at this concentration.

Pancreatic enzymes are secreted into the small intestine through the pancreatic duct and they are involved in digestion of proteins, carbohydrates, and fats in foods. As such, some studies have included the ability to tolerate the presence of pancreatic enzymes as another criterion for selection of probiotic cultures (Salminen, 1998; Ronka *et al.,* 2003). In this study, 3 h of exposure to pancreatic enzymes had little

TABLE 3
Viability of *Lactobacillus* strains after 3 h exposure to pH 3 and pH 7.2 (control)

Lactobacillus strain	Cell viability (log CFU/ml)[1]		Reduction in cell viability (log units)[1]
	pH 7.2	pH 3	
L. reuteri C10*	7.50±0.07	7.03±0.06	0.47 [a]
L. salivarius CI1	7.26±0.02	7.21±0.01	0.05 [b]
L. salivarius CI2	7.94±0.05	7.50±0.09	0.44 [a]
L. salivarius CI3	7.30±0.04	7.25±0.02	0.05 [b]

[1] Values are means ± SD of two independent experiments, each in triplicate
[a – b] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

adverse effect on the survival of the three *L. salivarius* strains (Table 5). All the three *L. salivarius* strains showed a very good tolerance to pancreatic enzymes (reduction in cell viability of 0.01 to 0.21 log units) and their tolerance levels were significantly ($P < 0.05$) higher than that of the commercial reference strain, *L. reuteri* C10 (reduction of cell viability of 0.46 log units). A similar result was reported by Ronka *et al*. (2003) who found that 3 h of incubation in growth medium containing pancreatic enzymes had little effect on viability of *L. brevis* strains. Ruiz-Moyano *et al.* (2008) also reported that 46 out of 51 tested lactic acid bacterial strains survived after 3 h of treating with 1.9 mg/ml of pancreatic enzymes.

Adherence Ability

Every potential probiotic strain is expected to attach to the epithelial cells of the intestine in order to colonize and establish in the intestine (Lee & Salminen, 1995). Furthermore, strong adherence to the intestine is necessary for releasing some probiotic bio-effects such as cholesterol lowering effects (Marteau, 2002), immune-modulation (Schiffrin *et al.,* 1995), and antimicrobial activities against pathogens (Mack *et al.,* 1999). In the present assay, adherence of the three *L. salivarius* strains to the Caco-2 cell line was in the range of 10 to 15 cells per Caco-2 cell (Table 6). *Lactobacillus salivarius* CI2 with an

TABLE 4
Growth of *Lactobacillus* strains in MRS broth (control) and MRS broth containing 0.3% bile salt

Lactobacillus strain	Cell viability (log CFU/ml)[1]		Reduction in cell viability (log units)[1]
	MRS	MRS + 0.3% bile salt	
L. reuteri C10*	8.28±0.03	7.82±0.06	0.46 [a]
L. salivarius CI1	8.24±0.03	6.55±0.37	1.69 [b]
L. salivarius CI2	8.24±0.03	6.81±0.30	1.43 [b]
L. salivarius CI3	8.28±0.09	6.67±0.41	1.61 [b]

[1] Values are means of two independent experiments, each in triplicate
[a – b] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

TABLE 5
Viability of *Lactobacillus* strains after 3 h exposure to 1.9 mg/ml pancreatic enzymes and normal condition (control)

Lactobacillus strain	Cell viability (log CFU/ml)[1]		Reduction in cell viability (log units)[1]
	Control	1.9 mg/ml pancreatic enzymes	
L. reuteri C10*	7.59±0.05	7.14±0.07	0.45 [a]
L. salivarius CI1	6.94±0.04	6.73±0.08	0.21 [b]
L. salivarius CI2	7.77±0.06	7.68±0.06	0.09 [c]
L. salivarius CI3	6.87±0.04	6.86±0.04	0.01 [c]

[1] Values are means of two independent experiments, each in triplicate
[a – c] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

TABLE 6
Adherence of cells of Lactobacillus strains to Caco-2 cell

Lactobacillus strain	Adhesion index (Lactobacillus cells per Caco-2 cell)[1]
L. reuteri C10*	10.5±0.1 [c]
L. salivarius CI1	13.1±0.5 [b]
L. salivarius CI2	15.3±0.4 [a]
L. salivarius CI3	10.3±0.0 [c]

[1] Values are means of two independent experiments, each in triplicate
Adherence was evaluated in 20 random microscopic fields
[a – c] Means within a column with different superscripts are significantly different ($P < 0.05$)
* Commercial reference strain

adherence ability of 15 cells per Caco-2 cell and *L. salivarius* CI1 with an adherence ability of 13 cells per Caco-2 cell showed significantly ($P < 0.05$) higher adherence ability than *L. salivarius* CI3 and the commercial reference strain *L. reuteri* C10, both with an adherence ability of 10 cells per Caco-2 cell. Similar findings were reported by Jacobsen *et al.* (1999) who studied 47 *Lactobacillus* strains for their ability to adhere to Caco-2 cells and found considerable variations, from strong to low adhesion, among the strains. Gopal *et al.* (2001) also found that *L. rhamnosus* DR20, *L. acidophilus* HN017 and *B. lactis* DR10 exhibited strong ability to adhere to the Caco-2 and HT-29 human epithelial cell lines.

CONCLUSION

Three bacterial strains isolated from the intestines of chickens were identified as *L. salivarius* using carbohydrate fermentation profile analysis by the API system and 16S rRNA gene sequencing. *In vitro* assays showed that all three *L. salivarius* strains exhibited good acid, bile and pancreatic enzyme tolerance, and good ability to adhere to Caco-2 cells. This indicated that all three *L. salivarius* strains would probably be able to survive, attach, and colonize GIT of chickens and could be considered as potential probiotic candidates for chickens. However, further *in vivo* studies in chickens need to be undertaken to evaluate the efficacy of the three *L. salivarius* strains in host animals.

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All submissions must be accompanied by a cover letter detailing what you are submitting. Papers are accepted for publication in the journal on the understanding that the article is original and the content has not been published or submitted for publication elsewhere. This must be stated in the cover letter.

The cover letter must also contain an acknowledgement that all authors have contributed significantly, and that all authors are in agreement with the content of the manuscript.

The cover letter of the paper should contain (i) the title; (ii) the full names of the authors; (iii) the addresses of the institutions at which the work was carried out together with (iv) the full postal and email address, plus facsimile and telephone numbers of the author to whom correspondence about the manuscript should be sent. The present address of any author, if different from that where the work was carried out, should be supplied in a footnote.

As articles are double-blind reviewed, material that might identify authorship of the paper should be placed on a cover sheet.

Peer review

Pertanika follows a **double-blind peer-review** process. Peer reviewers are experts chosen by journal editors to provide written assessment of the **strengths** and **weaknesses** of written research, with the aim of improving the reporting of research and identifying the most appropriate and highest quality material for the journal.

In the peer-review process, three referees independently evaluate the scientific quality of the submitted manuscripts. Authors are encouraged to indicate in the **Referral form** using the **Manuscript Submission Kit** the names of three potential reviewers, but the editors will make the final choice. The editors are not, however, bound by these suggestions..

Manuscripts should be written so that they are intelligible to the professional reader who is not a specialist in the particular field. They should be written in a clear, concise, direct style. Where contributions are judged as acceptable for publication on the basis of content, the Editor reserves the right to modify the typescripts to eliminate ambiguity and repetition, and improve communication between author and reader. If extensive alterations are required, the manuscript will be returned to the author for revision.

The Journal's review process

What happens to a manuscript once it is submitted to *Pertanika*? Typically, there are seven steps to the editorial review process:

1. The executive editor and the editorial board examine the paper to determine whether it is appropriate for the journal and should be reviewed. If not appropriate, the manuscript is rejected outright and the author is informed.

2. The executive editor sends the article-identifying information having been removed, to three reviewers. Typically, one of these is from the Journal's editorial board. Others are specialists in the subject matter represented by the article. The executive editor asks them to complete the review in three weeks and encloses two forms: (a) referral form B and (b) reviewer's comment form along with reviewer's guidelines. Comments to authors are about the appropriateness and adequacy of the theoretical or conceptual framework, literature review, method, results and discussion, and conclusions. Reviewers often include suggestions for strengthening of the manuscript. Comments to the editor are in the nature of the significance of the work and its potential contribution to the literature.

3. The executive editor, in consultation with the editor-in-chief, examines the reviews and decides whether to reject the manuscript, invite the author(s) to revise and resubmit the manuscript, or seek additional reviews. Final acceptance or rejection rests with the Editorial Board, who reserves the right to refuse any material for publication. In rare instances, the manuscript is accepted with almost no revision. Almost without exception, reviewers' comments (to the author) are forwarded to the author. If a revision is indicated, the editor provides guidelines for attending to the reviewers' suggestions and perhaps additional advice about revising the manuscript.

4. The authors decide whether and how to address the reviewers' comments and criticisms and the editor's concerns. The authors submit a revised version of the paper to the executive editor along with specific information describing how they have answered' the concerns of the reviewers and the editor.

5. The executive editor sends the revised paper out for review. Typically, at least one of the original reviewers will be asked to examine the article.

6. When the reviewers have completed their work, the executive editor in consultation with the editorial board and the editor-in-chief examine their comments and decide whether the paper is ready to be published, needs another round of revisions, or should be rejected.

7. If the decision is to accept, the paper is sent to that Press and the article should appear in print in approximately three months. The Publisher ensures that the paper adheres to the correct style (in-text citations, the reference list, and tables are typical areas of concern, clarity, and grammar). The authors are asked to respond to any queries by the Publisher. Following these corrections, page proofs are mailed to the corresponding authors for their final approval. At this point, only essential changes are accepted. Finally, the article appears in the pages of the Journal and is posted on-line.

English language editing

Pertanika **emphasizes** on the linguistic accuracy of every manuscript published. Thus all authors are required to get their manuscripts edited by **professional English language editors**. Author(s) **must provide a certificate** confirming that their manuscripts have been adequately edited. A proof from a recognised editing service should be submitted together with the cover letter at the time of submitting a manuscript to Pertanika. **All costs will be borne by the author(s)**.

This step, taken by authors before submission, will greatly facilitate reviewing, and thus publication if the content is acceptable.

Author material archive policy

Authors who require the return of any submitted material that is rejected for publication in the journal should indicate on the cover letter. If no indication is given, that author's material should be returned, the Editorial Office will dispose of all hardcopy and electronic material.

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Lag time

A decision on acceptance or rejection of a manuscript is reached in 3 to 4 months (average 14 weeks). The elapsed time from submission to publication for the articles averages 5-6 months.

Hardcopies of the Journals and off prints

Under the Journal's open access initiative, authors can choose to download free material (via PDF link) from any of the journal issues from Pertanika's website. Under "Browse Journals" you will see a link entitled "Current Issues" or "Archives". Here you will get access to all back-issues from 1978 onwards.

The **corresponding author** for all articles will receive one complimentary hardcopy of the journal in which his/her articles is published. In addition, 20 off prints of the full text of their article will also be provided. Additional copies of the journals may be purchased by writing to the executive editor.

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BACKGROUND

Pertanika began publication in 1978 as the Journal of Tropical Agricultural Science (JTAS).

In 1992, a decision was made to streamline *Pertanika* into **3 journals**. i.e.,

1. Journal of Tropical Agricultural Science (JTAS)
2. Journal of Science and Technology (JST)
3. Journal of Social Sciences and Humanities (JSSH)

BENEFITS TO AUTHORS

PROFILE: *Pertanika* publishes original academic articles rapidly. It is fully committed to the Open Access Initiative and provides free access to all articles as soon as they are published.

QUALITY: Articles submitted to *Pertanika* undergo rigid originality checks. Our double-blind peer review procedures are fair and open.

AUTHOR SERVICES: We ensure that your work reaches the widest possible audience in print and online rapidly. Submissions are through **ScholarOne** system by Thomson Reuters.

SUBMISSION GUIDELINES

The Journal accepts articles as **regular**, **short communication** or **review papers**.

The article should include the following:

- An abstract of not more than 300 words;
- Up to 8 related keywords;
- Name(s), Institutional affiliation(s) and email(s) of each author.
- The maximum length of your article must not exceed:
  - approximately 6000 words or 27 pages, including abstract, diagrams tables and references for full research papers,
  - 2000 words for short communication papers, or
  - 4000 words for review papers
- References should be listed in APA style.

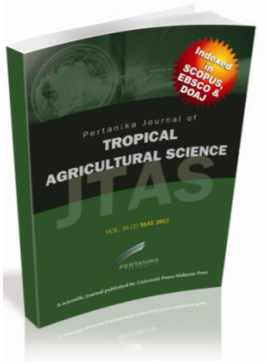
SUBMISSION DEADLINE

You may submit your articles at any time of the year. The journal is now accepting papers for its 2013-14 issues.

CONTACT US

For guidance on the submission process, or for any questions regarding submissions, you may contact the **Chief Executive Editor** at: nayan@upm.my

About the Journal



- An international multidisciplinary peer-reviewed leading Malaysian journal.
- Publishes articles in **English** quarterly. i.e., *February, May, August* and *November*.
- The elapsed time from submission to publication for the articles averages 5 to 6 months. A decision on acceptance of a manuscript is reached in 3 to 4 months (average 14 weeks).
- Indexed in **SCOPUS** (Elsevier), **Thomson (ISI) Web of Knowledge** [CAB Abstracts], **EBSCO**, **DOAJ**, **Agricola**, **CABI**, **Google Scholar**, **MyAIS**, **ISC** & **Rubriq**.

Scope of Journal

- Pertanika JTAS aims to provide a forum for high-quality research related to **tropical agricultural research** dealing with issues of worldwide relevance.
- Refer to our website for detailed scope areas. http://www.pertanika.upm.edu.my/scope.php

Format for Paper Submission

- Articles should include the following:
  - problem formulation
  - conceptual framework
  - methodology/ approach
  - research design (if applicable)
  - statistical analysis (if applicable)
  - main findings
  - overall contribution
  - conclusions & suggestion for further research
  - acknowledgements (if applicable)

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Contents

Pertanika Editorial Office, Journal Division
Office of the Deputy Vice Chancellor (R&I)
1st Floor, IDEA Tower II
UPM-MTDC Technology Centre
Universiti Putra Malaysia
43400 UPM Serdang
Selangor Darul Ehsan
Malaysia

http://www.pertanika.upm.edu.my/
E-mail: executive_editor.pertanika@upm.my
Tel: +603 8947 1622 / 1619

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http://penerbit.upm.edu.my
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