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NEPAL FOOD SCIENTISTS AND TECHNOLOGISTS ASSOCIATION

The Association is a professional and educational organization of Food Scientists and Technologists of Nepal with its central office in Kathmandu.

There are two chapters- Purbanchal (Eastern Development Region) and Narayani (Mid Development Region) of the association within the country which are located at Dharan and Hetauda respectively. Similarly, there are three chapters of the association in abroad situated in United States of America, Austrailia and Nigeria.

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- To facilitate the development and popagation of knowledge of Food Science and Technology.
- To provide a forum for discussion and exchange of the outcomes of research work in the field of Food Science and Technology.
- To create supportive environment in order to encourage Food Science and Technological innovations.

Major Activities

- Publication of Journal of Food Science and Technology Nepal (JFSTN), Food Nepal and Newsletters.
- Arranging lectures and seminars on different aspects of Food Science and Technology for the benefit of members and the Public

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Editorial

Out of all the resources required, human resource is the most important resource from the perspective of National development which significantly brings noticeable transformation in the economic development of a country. If we see the human resources situation of Nepal, it shows the challenging impression reflecting the need to deliver intensive efforts with high degree of priority guided by a well versed national policy. Being given with abundant biodiversity and good potential for agro-business, the propagation of agro-industries could not take the pace with time which otherwise contribute to the economy of the country by generating more employment and revenue with the production of export oriented products. The recent trends of migration of unskilled workforce outside from different location of the country has given new challenges on how to make them to retain in place providing employment locally based on the agricultural foundation within the indigenous circumstances.

With the aid of diversification in terms of climate and topography, the country is also rich in many of the community based traditional foods practically within the location and communities from the very long time. However, these traditional products are experiencing the diminishing trends because of the migrating trends of present generation to other jobs within or outside the country. Equally, the reasons of replacing indigenous foods are the modern processed products of imported pallets and taste. The storm of globalization has been engulfing the cultural products existed through out the country.

The situation urges that there is need of thorough review on the human resources development policy in the country especially focusing the local needs of skilled human resources based on the pre-harvest and post-harvest operational requirements including propagation of locally feasible food processing, packing and product development ventures. In doing so, the traditional community based food products are to be taken care for their technological upgrading giving due emphasis to their indigenous originality in flavor and taste. Furthermore specific requirement of human resources in agro food sector, it should also be envisaged to integrate with tourism and export trade of the country. In this regard, the need of continuous intervention has been intensely felt in the sphere of Food Science and Technology in the entire regime of the profession. So, it is the roles and responsibilities of we, Food Scientists and Technologists to make aware of the matter to policy level by contributing with the professional impacts to the system as a whole.

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Traditional Foods and Beverages of *Newari* Community – A Brief Review

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Newar is one of the popular ethnic groups having diverse cultural heritage and food habits in Nepal. Newars consume varieties of traditional both fermented and nonfermented foods and beverages. The major traditional fermented foods and beverages of Newari Community are Poko, Thwon, Ayella, Dhau and Khaya and the major non-fermented foods and beverages are Baji, Syabaji, Wo, Kwa-ghasa, Kwati, Yomari, Chatamari, Mee-kwa, Khen, Chhoyala, Kachila, Sukula, Lago, Lapi, Bhuttan, Momo, Takha and Sanyakhuna. This article presents the available informations on various traditional foods and beverages of Newari community on their cultural importances, preparation methods and consumption patterns.

Keywords: Traditional foods, Cultural practices, Newar, Preparation, Consumption, Nepal

Introduction

Nepal is a country of different ethnic groups, cultural habitats, and indigenous food heritage. There are 59 ethnic groups and over 75 different dialects (Manandhar, 1997). *Newar* is one of the major popular ethnic groups of Nepal having rich diverse cultural heritage and food habits. They are the original inhabitants of Nepal and are scattered in the main cities like *Kathmandu, Dharan, Dhankuta, Bhojpur, Chainpur, Narayanghat, Hetauda, Banepa, Tansen, Bandipur, Dolakha, Butawal, Doti* covering all regional areas throughout the country. They have Mongolian features and their own language and script. *Newar* believed to have its origin from Tibeto- Burmans having complex social system and practices tend to live in communal settings. According to Nepal census (2001), there is 1,245,232 population of *Newar* in the country, occupying the nation's sixth largest ethnic group representing 5.48%.They produce wide varieties of foods and beverages with an astounding range of tastes and flavors. *Newars* are very famous in celebrating festivals and varied cultural occasions. Dishes used in feasts are of full nutrient content such as protein, carbohydrate, fat, minerals, vitamins as well as probiotics (fermented food). The main categories of agricultural materials used as traditional cultural foods and beverages are cereal and cereal products, pulses, egg, fish, meat products, vegetables and milk products.

Local knowledge and beliefs transmitted from generation to generation, and the dynamic resources modified by contemporary experiences and experimentations are leading the developments in traditional foods and beverages in *Newari* community. Though technologies are primitive, they have played a major role in the economic status of *Newari* people. However complete scientific informations on these various foods and beverages products, their traditional ethics, production and preparation methods and mode of consumption of these products are lacking. This article aims to explore the available informations on various traditional foods and beverages of *Newari* community.

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Consumption pattern of Foods and Beverages in *Newari* Community

Newari community has speciality in food consumption pattern. They use biodegradable materials such as *Dena lapte*, *Dona*, *Salincha* and *Pala* for the service of foods and beverages. *Dena lapte* is a kind of plate made up of *Sal* leaves, *Dona* is a type of bowl made of *Sal* leaves, *Salincha* is made up of mud and used for *Raksi*. Similarly *Pala* is made up of mud and used for liquid food. The food preparations, consumption patterns and materials used for food consumption in *Newari* ethnicity seems scientific and skilled although study on various traditional foods and beverages is limited. People are unaware of such indigenous practices and not exploring the tradition due to the lack of scientific study.

Newari ethnicity consumes various traditional food and beverage products. Food products like *Samay baji* is also offered to God. *Samaybaji* includes *Baji, Syabaji,* roasted black soyabean, boiled marinated cowpea, pieces of ginger, *Chhoyala*, small fishes like *Ghinge macha* or *Kunya*, boiled egg (duck or hen), *Wo* (*Bara*), *Thwon*, *Ayella* and *Dhau*. They offer *Samaybaji* to Lord Shiva in *Paanchare* day which falls on the day of April/ May.

Newars consume feast during celebrating festivals and on cultural occasions. The feast includes various fermented and non-fermented food and beverages products which should be served traditionally in systematic way. The feast is performed sitting people in the ground serially from elder to younger. The common cultural practice of the service of *Newari* foods and beverages is given in Fig 1. Those foods and beverages are generally served in plates and bowels made of *Sal* leaves called *Denalapte* and *Dona* respectively.

Achar → *Baji* → *Mee-Kwa* → *Chhyala* → Vegetable Curry → Meat products (Meat *curry, Takha, Sanya khuna, Chhoyala*) → *Thwon* → *Ayella* → *Bhuttan* → *Tunka* → *Paun-Kwa* → *Dhau* → *Sisa busa* → *Gway* (Bettle nut) *and Lwang* (Clove) at last.

Fig. 1: Order of Serving Foods and Beverages of *Newari* Cummunity

The *Newari* traditional foods and beverages can be broadly divided into two groups i.e., Fermented and Non-fermented. Cultural importance, preparation methods and consumption patterns of the various traditional foods and beverages of *Newari* Community is presented in this article.

Traditional Fermented Foods and Beverages of *Newari* community

Fermentation is an age old process immemorial. Generally, the traditional methods of manufacturing these fermented foods and beverages are simple and can be prepared in home scale. If the manufacturing procedures are properly followed, the foods and beverages are usually safe for consumption (Rose, 1982).Traditional fermented foods and beverages are specific to certain geographic regions and also specific to the particular communities (Dahal *et al*., 2005). Fermented food and beverage products of *Newari* ethnicity are alcoholic beverages such as *Poko, Thwon, Ayella, Dhau and Khaya* (Table 1).

Mana and Manapu

Mana and Manapu are *Newari* words (*Murcha* in Nepali word) for the starter cultures used for the alcoholic fermentations. *Murcha* starters have been extensively used for preparing various kinds of traditional fermented foods and alcoholic beverages from starchy substrates, predominantly of rice. Available literature suggests that these starters includes starch hydrolyzing microorganisms like *Rhizopus spp., Mucor spp., Endomyces fibuligera*, and alcohol producing yeast *Sacharomyces cerevisiae*, and lactic acid producing bacteria (Heseltine *et al*., 1988). The lesson of *Murcha* preparation is confined mostly to daughter-in-law for maintaining technical secrecy within the family members of *Newari* community.

Mana and Manapu has been used for making traditional alcoholic beverages like *Thwon, Ayella and Poko* which have both ritual and entertaining values as well. These products have several symbolic significance in special occasions like marriage ceremony, in addition to offering alcohol to god and goddesses during holy festivals of alcohol drinking communities (Kunwar, 1984). *Manapu* is prepared in the form of round cakes usually from rice. *Mana* lumps possessing green color like *Koji*, is prepared from wheat. *Manawasha* (a kind of tree of fern spp.) is added to both *Mana* and *Manapu* during fermentation (Dahal *et. al.,* 2005; Karki & Shrestha, 1999; Gajurel & Baidya, 1979d).

Traditional manufacturing process of *Mana* and *Manapu* is as follows:

Mana is normally prepared locally from wheat or rice. Now a days, it is commercialized and available in market. Wheat is soaked for several hours, drained, steamed, and cooled. The mash is mixed with *Murcha* seed and *Manawasha,* kneaded to prepare cakes and then it is allowed to ferment by covering straw mat. Green molds appear on the cake which indicates the completion of fermentation (Dahal *et. al.,* 2005; Karki & Shrestha, 1999).

Manapu is prepared locally in the traditional style using rice flour or millet flour and other ingredients. Flour of rice or millet is taken and mixed with *Murcha* seed and *Manawasha*. The flour is kneaded to prepare cakes. The cakes are then placed on straw, allowed to ferment, and dried in sun to prepare *Manapu* (Dahal *et. al.,* 2005; Karki & Shrestha, 1999).

Microbial evaluation of traditional *Murcha* starter showed that the lactic acid bacteria and yeast are dominant at $5X10^5$ to $1X10^9$ cfu/g ranges while fungi were present at $2X10^5$ to $1X10^7$ cfu/g (Shrestha *et al.*, 2002).

Yeast and lactics are present in high numbers in *Manapu,* where as molds were dominant in wheat based *Mana* samples (Shrestha *et al*, 2002). In general *Manapu* starters based on the rice and millet showed a predominance of yeast and lactic in the range of $5X10^5$ to $1 X10^9$ cfu/g. The *Mana* starters contained molds as dominant flora recording more than $1X10^7$cfu/g. Among the yeast, *Saccharomyces cerevisiae* strains were found to be dominant followed by *Candida versatilis*. Among the lactics, *Pediococcus spp.* particularly *P. pentosacaus* was predominant. *Rhizopus spp* was dominant among molds (Shrestha *et al*., 2002).

Poko

Poko is rice based solid fermented food product characterized by creamy color, soft, and juicy sweet and sour taste with slightly alcoholic and aromatic flavor. Consumption of this product is deeply rooted in the culture and has several symbolic significance. During the preparation of *Thwon* and *Ayella,* this is the base product. There is a traditional belief that *Poko* promotes good health, nourishes the body giving good vigor and stamina (Gajurel & Baidya, 1979c). Their production is confined to home scale till to-date.

Traditional method of *Poko* production is as follows: Rice is soaked overnight, steamed until cooked, and sticky, spread to cool on the clean floor at room temperature, powdered *Manapu* is sprinkled on the rice, mixed well and packed in earthen vessels. The mouth of vessel is closed with leaves and straw and tied with muslin cloth. The whole vessel is then covered with a blanket or cloth to keep it warm and kept on straw mat and allowed to ferment at ambient condition for two or three days in summer and up to five days in winter. The product is mixed everyday during fermentation and the sticky rice is transferred to *Poko.*

Table 1 : Major Traditional Fermented Foods and Beverages of *Newari* community

Raw material	*Product type*	*Local name of the product*	*Nepali name*	*Mode of preparation*	*Consumption*
Cereal based	Starter culture	*Manapu* ((Dahal *et. al.*, 2005; Gajurel & Baidya, 1979d)	*Murcha*	Wild plant mixing and fermentation	For alcoholic fermentation
	Starter culture	*Mana* ((Dahal *et. al.*, 2005; Gajurel & Baidya, 1979d)	*Murcha*	Wild plant mixing and fermentation	For alcoholic fermentation
	Rice	*Poko* (Shrestha & Rati, 2003)	-	Fermentation	As energetic food
	Rice	*Thwon* (Gajurel & Baidya, 1979c)	*Jand*	Fermentation	Alcoholic soft drinks
	Wheat	*Ayella* (Gajurel & Baidya, 1979c)	*Rakshi*	Fermentation and distillation	Alcoholic hard drinks
	Millet	*Ayella* (Gajurel & Baidya, 1979c)	*Rakshi*	Fermentation and distillation	Alcoholic hard drinks
Milk based	Milk	*Dhau* (Majpuria, 2000)	*Dahi*	Fermentation	Feast, Ready to serve
	Milk	*Khaya* (N.R.)	*Mohi*	Dahi mixed with water and spices	Feast , Ready to serve liquor
Vegetable based	Raddish, Cucumber	*Achar* (N.R.)	*Achar*	Fermentation	As a delicious dish

(Note: N.R. = Not reported)

Microbial study of *Poko* product shows that *Sacharomyces cerevisiae, Candida versatilis, Lactobacillus sps, Pediococcus, Rhizopus sps* were the most dominant microorganisms identified (Shrestha *et al.*, 2002).

Poko has nutritional value. One of the study showed that *Poko* is rich in proteins, carbohydrate and vitamins. The increase in pyridoxine, thiamine, vitamin B_2, and niacin in *Poko* prepared using traditional *Manapu* starter cakes and defined starters as compared to the raw material used was found in the range of 50 and 59 %, 16 and 32 %, 18 and 53 %, 173 and 117 % respectively. The increase in Folic acid in *poko* product was up to 76 % (Shrestha *et al.*, 2003).

Thwon

Thown is a *Newari* word of *Jand*. *Thwon* is wort of *Poko a*nd a popular traditional alcoholic product in *Newari* community. Various ethnic groups of Nepal prepare *Jand* according to their own methods. It can be prepared in home scale. *Newari Jand i.e. Thwon* is clear and white in color. Some times *Thwon* is milky but tasty. *Newari* farmers (*Jyapu*) enjoy this refreshing drink which gives the constant companion as they work in the field or as they stay indoors (Majpuria, 2000).

The normal period for *Thwon* to be ready from start to finish is 4 days. It remains sweet for a couple of days. From on 6th to 7th day, it begins to get more bitter. f:*Newari* people, however, prefer to drink a mixture of the juice and *Thwon* which is known as *Bhyabar*. *Bhyabar* is considered to have the strong intoxicant property.

The process of preparation of *Thwon* is as follows: About 8 tea cups of rice is taken, soaked with water, cooked and spread on a bamboo tray *(Nangło)* until it cools down. In the summer season the rice should get absolutely cold but in winter the rice should remain a little warm. One piece of *Murcha* (*Manapu or Mana*) is taken and crushed in to small pieces and then mixed with the rice. The mixture is kept in a warm place and allowed to ferment generally for three to four days. The rice smells pleasant fermented flavour. It is then kept in a big jug and equal amount of water is added. In four or five hours, the water will extract the essence from the fermented rice. The liquid extract is called *Thwon* (Majpuria, 2000).

Detailed study on *Thwon* is lacking. But one study of rice based solid fermented food product called *Poko* showed that there was not much variation in Mesophilic aerobic count, which ranged from 1.3X10^7 to 7.9 X 10^7 cfu/g during 5 days of fermentation. However molds counts were lowered, between 6.3 X 10^5 to 1 X 10^6 cfu/g and even less that 1.3 X10^3 after the fourth day of fermentation. An increment of about one order of magnitude from 6 X10^6 to 5 X 10^7 cfu/g in lactic acid bacteria was perceptible. With yeasts, the counts showed a steady increase from 1.8 X10^6 to 1.3 X 10^8 cfu/g. pH dropped from 4.3 to 3.0, where as acidity increased steadily from 0.2 to 1.7 % as lactic acid. The reducing sugar and total sugar between 2 to 3 days of fermentation ranged from 14.4 to 15.6% and 14.6 to 18.2%, respectively. The highest organoleptic score was recorded on the second and third day's fermentation (Shrestha *et al.*, 2002). Typically, *Jand* contains 5-7 % alcohol, 0.8 – 1.1% acidity (as lactic acid), 1.6-2.5 % reducing sugar (as glucose), 1.6- 2.8 % total sugar (as sucrose), 12-14 % starch,

76-80% water and traces of methanol, esters, aldehyde and other flavor components (Upadhya, 2005).

Thwon finds a very prominent position in *Newari* culture. The tradition of offering *Thwon* to god and guests, during feast is a unique way of showing hospitality. It is also used in several festive occasions, ritual rites, settling deputies and appeasing deities. *Thwon* is considered as energetic food by the *Newari* community (Majpuria, 2000).

Ayella

Ayella is *Newari* word for *Raksi*. *Raksi* is a major traditional alcoholic beverage of *Newari* community. *Raksi* is an unaged generic spirit obtained by pot distillation of the slurry of *Jand*. The product likens whisky has highly varying alcohol contents generally between of 15 to 40 % (K.C *et al.*, 2004).

Ayella is obtained by distillation of fermenting mash of rice, millet and barley. Fermentation pattern is similar in case of *Thwon*. After fermentation, the mash is steam distilled in a traditional distillation apparatus. The apparatus generally contains three sections – bottom, centre, and top. The fermenting mash is placed in the bottom section and the apparatus is set over a fire. In the middle section, there is a distillate – collecting bucket, and at the top, the cold water pot for condensing alcohol vapor. Generally, the cold water pot is replaced by new cold water three times during distillation and the collected distillate in this period is called *Tinpane Raksi*, which is relatively high in alcohol (Gajurel & Baidya, 1979c). It may contain 20-40 % (v/v) alcohol (Karki, 1986).

Ayella prepared from millet (*Kodo)* is considered to be of good quality. *Ayella* is used as appetizer or medicine for stomach pain. It is believed that it gives strength and energy. *Ayella* is essential product for offering god in festivals.

Dhau

Dhau is a *Newari* word for *Dahi (*Yoghurt*). Dhau* is very popular fermented food of Nepal. In *Newari* culture, *Dhau* has great importance i.e. it is must in every cultural occasion, festivals, as well as in *Sagun.*

It is considered that *Dhau* conduces sound health and long life. Scientists believe that *Dhau* reduces the cholesterol level, is good for intestinal disorders and helps digestion. *Dhau* acts as a neutralizer agent for hot and heavily spiced dishes.

Dhau is generally prepared from the boiled cows or buffalos milk by inoculating with a small quantity of starter *Dhau*. Among different types of *Dhau* prepared, *Juju Dhau* is the most famous delicious, sweet and flavored *Dhau* prepared in Bhaktapur district. It has great demand due to its unique texture, taste, and flavor than other types of *Dhau* (Karki, 1986).

Khaya

It is consumed in feast and offered to God also. *Khaya* is prepared by adding water in *Dhau* mixing with salt, cumin powder, and other spices. This is diluted *Dhau*. There is a traditional belief that it makes body cool and nutritious also.

Achar

Achar is *Newari* word for pickles. *Achar* also refers to the pickle in Nepali. It is prepared by mixing of spices and salt with the pieces of different types of vegetables locally available like Raddish and Cucumber. The mixed product is kept in clean bottle and consumed after one week.

Traditional Non Fermented Foods and Beverages of *Newari* community

Newari community use varieties of non fermented foods and beverages. It consists of non-vegetarian and vegetarian items. Non-fermented foods and beverages include most of the food commodities nutritionally very rich (Table 2).

Cereal and cereal products

Baji / Syabaji

Baji is made up from paddy and used as main base dish in case of *samyabaji* and feast, where as *syabaji* is roasted *Baji* used in *Samyabaji*. These products can be stored for long time and are ready to eat products (Gajurel & Baidya, 1979a & 1979b).

In *Newari* community, *Baji* is prepared by soaking paddy (2-3 days) in water, boiled until small cracks appear, cooled, drained the excess water, dried, and roasted in *Handi* until it starts to puff. It is then transferred to an *Urghal*, and beaten by *Luci* with occasional shaking by *Luciku* for 15-30 minutes, husk is removed and flaky shape of *Baji* form. It is then shifted to a *Nanglo* and then the husk is removed to get *Baji* (Gajurel & Baidya, 1979a & 1979b).

Syabaji has culturally great role in *Newari* ethnicity. *Syabaji* is used in *Samya baji* the food offer to god and it is essential. *Syabaji* is prepared by roasting *Baji* in a *Handi* over fire with hot sand. *Baji* changes to *Syabaji* after putting in the hot sand by occasional shaking. The mixture is then transferred to *Nanglo,* and sand is removed by sieving in *Chalni* to obtain *Syabaji. Baji* expands considerably by two to three times during roasting (Gajurel & Baidya, 1979b).

Yomari

This is unique bread prepared by *Newari* people, on *Yomari punhi*, day which is observed on the full moon day in December, January (Majpuria, 2000). On this day, *Newars* prepare *Yomari*- a kind of conch shape bread made of rice flour, with solidified sugar (*chaku* with ground sesame seeds) inside. The shape of the bread resembles a Fig fruit which is called *Yomari* in *Newari*. It is quite tasty and people enjoy it. Laxmi, the goddess of wealth as well as *Bhakari* (Container made from bamboo to keep paddy) is worshipped on the day

Table 2: Major Traditional non-fermented Foods and Beverages of *Newari* community

Raw materials	*Product type*	*Local name*	*Nepali name*	*Mode of preparation*	*Consumption*
Cereal and cereal products	*Taichung* rice	*Baji* (N.R)	*Cheura*	Parboiling , roasting, flaking	Ready to serve
	Taichung rice	*Syabaji* (Gajurel & Baidya, 1979b)	-	Parboiling and roasting	Ready to serve
	Taichung rice flour	*Yomari* (N.R)	-	Steming	Ready to serve
	Taichung rice or other rice flour	*Chatamari* (N.R)	-	Making like Roti/Chapati	Ready to serve
Legumes	Black gram	*Wo* (N.R)	*Bara*	Soaking, grinding and fat frying	With samyabaji
	Black soyabean	*Haku musya* (N.R)	*Kalo bhatmas*	Roasting	With samaybaji
	Fenugreek and small pea	*Mee- Kwa* (N.R)	*Methi ra sano kerau*	Cook like curry	During feast
	Nine types of Pulses	*Kwati* (Majpuria, 2000)	*Nau Geda Gudi*	Cook like dal or curry with lots of soup	During festival
	Mixed dishes	*Kwa- Ghasa* (N.R)	-	Dishes mixed and cooked	During festival as main dish
Egg/ Fish	Egg	*Khen* (N.R)	*Anda*	Boiled and fried	During festival and in Sagun
	Fish	*Nyaa/Sanya/ Kunya* (N.R)	*Machha*	Small fish/ fried fish	During festival and in Sagun
Meat	Buff meat	*Sukula* (N.R)	*Sukuti*	Sun dried, smoked and marinated with spices	As snacks food
	Buff meat	*Kachila* (Shrestha *et al.*, 1999)	-	Keema mixed with heavy spices	Snack food or in feast
	Buff meat	*Chhoyala* (Shrestha *et al.*, 1999)	-	Boiled and marinated with spices	Snack food and in feast, Samaybaji
	Buff meat	*Momo* (Shrestha *et al.*, 1999)	-	Steamed	Snack food
	Buff	*Takha* (Shrestha *et al.*, 1999)	-	Jellied meat	During festival and cultural occasions
	Soup of buff meat and fried dry fish	*Sanyakhuna* (Shrestha *et al.*, 1999)	-	Jellied fish soup	During festival and cultural occasions
	Buff meat organs	*Bhuttan* (N.R)	*Bhuttan*	Boiled and fried	During festival and as snacks food
	Buff keema	*Lago/Lapi* (N.R)	-	Steamed and fried	During festival and feast
Vegetables	Potato and bamboo shout	*Chhyala* (N.R)	*Tama aloo*	Cooked as curry	During festival and feast
	Green mustard	*Tunka/ waaunchha* (N.R)	*Tori ko sag*	Boiled and marinated with spices	During festivals, and in Samaya baji
	Garlic leaves	*Laba* (N.R)	*Lasun*	Raw garlic leaves marinated with spices	During festivals, and in Samaya baji, Snack food
Miscellaneous	Rice flour and black gram flour	*Lakhamari* (N.R)	-	Deep fried	During cultural occasions, festivals
	Wheat flour (*Maida*)	*Ayantha-mari* (N.R)		Deep fried	During cultural occasions, festivals
	Whole Wheat flour (*Anta*)	*Math* (N.R)	-	Deep fried	During cultural occasions, festivals
	Lapsi powder	*Pau kwa* (N.R)	-	Sour Soup	During cultural occasions, feast
	Raw cucumber, small pea and radish	*Sisa busa* (N.R)	-	Pieces of raw cucumber, small pea, and radish	During feast

(Note: N.R = Not reported)

with *Yomari* of different shapes such as lady with *Nanglo, Kucho*, Ganesh, Mouse, Tortoise, Dog, *Mayo* and *Bayo*. *Yomari* is kept inside the heap of paddy.
Yomari is prepared by kneading rice flour using hot water, making Fig shaped, making hole and putting solidified *Chaku* mixed with powder of fried black sesame. The hole is closed and made flower like shape and steamed until cooked. It is served while hot.

In the festival, gods are worshipped for good harvest for paddy. The blessing of gods is sought so that family may not face the shortage of rice. It is believed that rice bread for the harvest moon i.e. *Yomari* helps family to remain unexhausted. *Yomari* is also prepared in other celebrations.

Chatamari

This is a rice flour pancake popular among *Newars*. In the month of April/ May, *Dewali* or *Degudeopuja* is celebrated. *Newars* prepare *Chatamari* to offer to *Devta* named *Degudeo* or *Kuldevta* (festival god) and distributed as Prasad (offering). During *guthi* (Religious trusties) feasts, *Chatamari* are prepared as an important item for ritualistic worshipping (Majpuria, 2000).

Legume/Pulses Products

Wo

Wo is very popular dish of *Newari* culture essential for feast as well as offering *Samyabaji* to god. *Sithi nakha* (*Kumar sasthi)* which falls on the mid May/June is special festival day for preparation, offering god and consumption of *wo* (green gram or black gram).

The traditional method employed in the preparation of *Wo* is as follows: Black gram is splitted in *Janto*, cleaned, soaked in water overnight, and husk is removed by hand washing 2 to 3 times in water. The wet dehulled black gram is then ground in a *Silauta* (Now days most people use grinder), small water is added to get thick paste. Thick paste is mixed well by hand. Small amount of salt, ginger juice and small piece of *Hing* is added. In *Tawa,* some oil is put, heated and then small ball of black gram paste is made. Hot oil is put, spread the black gram paste by finger and made thin round *Roti*, fried both side until it becomes brownish. This product is slightly similar to *Fulaura* (Karki, 1986), but the structure and cooking procedure is different.

Wo can also be prepared by using green gram instead of black gram. *Keema* can be put at the top of *Wo*, spread and cooked. There is a tradition that green gram *Wo* is given to sick people for energy. *Wo* is fabulous, tasty and nutritious.

Haku-musya

Haku musya is a product of black soybean. Black soybean is roasted, mixed with little salt and oil to prepare *Haku musya*. This product is essential part of *Samyabaji.*

Mee-Kwa

This dish is very popular in *Newari* community of Patan (Lalitpur district). *Methi* (Fenugreek) and *Sano kerau* (small pea) are soaked in water separately in the ratio of 1:2 for 24 h, cleaned by hand washing two to three times. *Methi* should wash more time than small pea to get rid of turbid taste. *Methi* and small peas are mixed, fried and cooked as other vegetables adding necessary spices. In some *Newari* community, *Methi* is germinated. There is traditional belief that *Mee- kwa* is good for health.

Kwati

This preparation is made especially on *Janai purnima* or *kwati purnima* which falls on the full moon day of *Shrawan* (July/ August). This dish is very delicious and nutritious and is made usually from nine types of whole seeds of pulses *(gedagudi*) and beans viz. Small field pea, Large field pea, *Simi*, Cow pea, *Bakula, Masyang,* Soyabean, Bengal gram and Black gram. This is prepared by soaking pulses in water for 24 h (Most of *Newars* sprouts it) and cooked well adding water and spices. *Momo* and pieces of *Roti* can be added. Oil is heated in pan, *Jwano* is fried and the fried *Jwano* and heated oil is added to *Kwati* (Cooked pulses soup). In Lalitpur district, there is one plant called *Laincha ghain*, which is added to *Kwati.* It is served hot and used as beverage in the evening of *Janaipurnima.* It is supposed to give vigorous heat to the body.

Kwati is most nutritious among *Newari* diets. It provides calcium, phosphorus, iron, and vitamin B. It is particularly a rich source of proteins for vegetarians (Majpuria, 2000).

Kwa-ghasa

Kwa ghasa is a mixed dish of all food commodities such as cereals, pulses, meat products, egg, fish, vegetables, mushroom, spice and alcoholic beverages. *Kwa ghasa* is prepared during *Tihar* festival. Goddess Laxmi is offered by *Chaurasibyanjan* i.e. Eighty four types of foods and beverages available in the market. *Chaurasibyanjan* are kept in *Thayavu (*Bronze plate). All the food available in market such as *Baji, Syabaji*, different types of pulses, vegetables, mushroom, meat products, egg, fish, *Wo, Dhau,* alcoholic beverages are kept at banana leaf in *Thayavu* and offered to goddess *Laxmi*. The product kept in *Thayavu* is called *Prasaad. Prasaad* is taken out next day of *Bhai tika*, mixed well and cooked once more with lots of oil, spices, garlic, and ginger for delicacy. After cooking, this dish becomes thick soup with solid particles. According to *Newari* custom, *Prasaad* should be taken out by own family members only. This dish is outstanding in taste and flavor, very nutritious and all *Newars* love it.

Egg & Fish products

Newars are mostly nonvegeterian. Meat products, egg and fish products are essential during worshipping, festival, and cultural occasions.

Khen

Khen is a *Newari* word for Egg. Boiled and fried egg has great importance in *Newari* culture. *Khen* is used as *Sagun i.e.* Food materials represents as moon which is for a good luck (Majpuria, 2000). Food is offered as *Sagun* which confers good luck and protects against evils. *Sagun* includes egg, fish, meat, ginger pieces, *Wo, Dhau*, alcohol in *Puja thali*. Egg is also essential parts of *Samaybaji*.

In Kathmandu, egg is used during religious ceremonies. They offer eggs to various gods and goddesses including Ganesh (Elephant headed deity).

Nyaa/Sanya/Kunya

Small fish (or *Nyaa*) is essential parts of *Sagun* and *Samaybaji* in worshipping, festivals and cultural occasions. Fried fish are called *Sanya and Kunya*. These products are nutritious and delicious.

Meat products

Buff meat is delicacy among *Newars*. They make several delicious preparations of buff such as *Kachila, Chhoyala, Momo, Takha, Sanyakhuna, Sukula, Bhuttan, Lago, and Lapi*.

Sukula

Sukula is *Newari* word for *Sukuti* i.e., Dried meat in Nepali language. Dried meat is very popular among *Newari* community. Buff meat cleaned, cut in to small slices and pieces, mixed with spices, salt, oil, and sun dried until brittleness, and fired, mixed with spices and served.

Microbial examination of 30 samples of *Sukuti* showed that total viable count ranged from 10^1–10^5 cfu/g. *Coliform* was found in 13%, mold in 30%, *Staphylococcus aureus* in 13.3%, *Salmonella in* 3% where as *Clostridium perfringens* and yeast were absent in all samples (Shrestha, 1999).

Kachila

Kachila is raw Buff *Keema*. Buff meat is cleaned, grounded and mixed with heavy spices, salt and hot oil with fenugreek. It is now ready to serve. This dish is consumed as snack food or in feast.

Microbial study of 42 samples of *Kachila* showed that total viable count were more than 10^4 cfu/g. *Coliform* was found in 95% of sample, Yeast and mold found in 42% of sample, where as 4.7% of sample showed *Salmonella* positive and 11.9% of sample showed *Staphylococcus aureus* positive. *Clostridium* was absent in all samples. This might be due to the unsatisfactory sanitation and handling practices (Shrestha *et al.*, 1999).

Chhoyala

Chhoyala is a very popular meat dish of *Newari* community. This product is used for worshipping god, festivals, and cultural occasions as well as in other get together programs as snack food. *Chhoyala* is prepared as follows: Buff meat is cleaned and boiled until cooked. It is then cut into small pieces and mixed with spices. Oil is heated, red chilly and methi is fried separately, and mixed with cooked meat. *Chhoyala* is now ready to serve.

Microbial examination of 51 sample of *Chhoyala* showed that total viable count was found to be less than 10^3 cfu/g. *Coliforms* were found in 62% of sample, yeast and mold in 33%, *Staphylococcus aureus* in 7.8% of sample and *Salmonella* in 1.9%, whereas *Clostridium* was absent in all samples. Though *Chhoyala* is cooked optimally, contamination may occur due to the negligence in sanitizing utensils, personnel hygiene, dust, air, and use of unhygienic water after preparation (Shrestha *et al.*, 1999).

Momo

It is popular delicacy of *Newari* ethnicity. Now a days, *Momo* hits the streets of Kathmandu and other places of Nepal. These have become very popular even with *Bhramins* and *Chhetri* community. It is mostly eaten during winter season. Now days, it is available in all the season. It is usually prepared by buffalo meat. However, other types of meat can be substituted in place of buffalo meat.

Momo is prepared by making small round shaped dough, spiced *Keema* is put on the centre of the dough, making the shape of a flower, steamed properly and served hot with *Momo achar* (tomatoes spices, salt and sesame).

Microbial examination of 29 samples of raw *Momo* showed that Coliform was found in all samples. Yeast and mold count, -*Staphylococcus aureus* and *Salmonella* were found in 52%, 68% and 6.8% of samples respectively. *Clostridium* was absent in all samples. This contamination might be due to unhygienic raw materials used (Shrestha *et al.*, 1999).

Where as in case of cooked *Momo*, out of 29 samples, the microbial study showed that *Coliform* was found in 17% of samples, yeast and mold count in 3% whereas *Salmonella, Staphylococcus aureus* and *Clostridium perfringens* were absent in all samples. Out of 23 samples of *Momo achar*, Coliform and yeast & mold were found in 34% and 8.6% of samples respectively. Pathogenic bacteria like *Salmonella, Staphylococcus aureus*, and *Clostridium* were absent in all the samples (Shrestha *et al.*, 1999).

Takha

Meat jelly of buff (*Thalthale Masu/ Takha*) is especially peculiar to *Newar*. This type of meat is prepared in large scale for big feasts and on marriage occasions. Such meat is prepared only in winter and can be preserved for at least two week with out keeping in refrigerator.

Takha is prepared by cooking buff meat (including skin part and head part) properly with spices, salt with adding lots water until liquid part shows sticky ness. Put in clay pot (*Bhegat*), or now day's people use steel flat pot like utensils (*Ari*).Heat spoon of oil, add big cumin and red chili. Add these products to the jelled dish and left for overnight. Next day dish becomes fully jelled. It is called *Thalthale Masu*.

Microbial study of 13 samples of *Takha* showed that 53% of sample showed *Coliform* and 23% of sample showed yeast and mold count (Shrestha *et al.*, 1999). Pathogenic bacteria like *Salmonella, Staphylococcus aureus*, and *Clostridium perfringens* were absent in all samples of *Takha*.

Sanyakhuna

This is a kind of very spicy, hot and sour *Takha* with fried fish mixed. This dish is very delicious and has unique tastes. The process of preparing *Sanyakhuna* is as follows : Liquid part of cooked *Takha* is taken, fried fish (*Ghinge machha* which has very strong smell), ginger, garlic paste, spices, salt, red chilli powder are added. It is boiled for few minutes, cooled and a spoon of lemon or *Kaljyamir* juice is mixed. It is then put in clay pot (*Bhegat*). Now a days, people use steel flat pot like utensils (*Ari*). A spoon of oil is heated separately, big cumin and red chillies are added to the hot oil. This product is then poured on the surface of jelled dish and left overnight. Next day, dish becomes fully jelled. It is called *Sanyakhuna* (*Sanya i*s fish in *Newari* language and *Khuna* means cooked). This product has fabulous taste.

Microbial study of 10 samples of *Sanyakhuna* showed that *Coliform* was present in 50 % of samples. 40% of sample showed the presence of yeast and mold. Pathogenic bacteria like *Salmoneila, Staphylococcus aureus*, and *Clostridium perfringens* were absent in all samples. *Takha* and *Sanya khuna* both are well cooked products. Contamination of *Coliform* and yeast and mold might be due to unhygienic handling practices as well as the use of clay pot to jell the product (Shrestha *et al.*, 1999).

Bhuttan

Bhuttan is used in feast as snack food with hard drinks. It is prepared from the portion of liver, intestines, lungs, hearts, etc either of buffalo or goat. These portions are boiled until cooked, cut in to small pieces and deep fried. Spices are mixed and it is ready to serve. This dish is tasty and delicious (Majpuria, 2000).

Lago / Lapi

In *Newari* language, *La* means meat and *Go* means ball indicating that *Lago* is a product similar to meat ball. It is used in *Tihar* for *Chaurasibyanjan*. *Lago* is prepared by mixing salt, spices and oil to buff *Keema* and it is moulded to make meat balls. These balls are steamed and fried to get *Lago*.

Lapi is a round bread prepared from *Keema* (*La* means meat and *Pi* means flat round). Method of preparation and consumption of *Lapi* is similar to *Lago*.

Miscellenous products

Chhyala/Tunka/Waaunchha

Chhyala is popular vegetable dish of *Newari* community. Vegetables like potato, bamboo shoot, cow pea, dried radish pieces are cooked together as curry with enough soup. The cooked product is *Chhyala* which is delicious. *Tunka* and *Waaunchha* are boiled green mustard leaf marinated with spices and salt. These products are consumed during festivals.

Lakhamari/Ayanthamari/Math

Lakhamari is a type of sweets of *Newars*. It is made up of flour, sugar, butter etc. It is made in different shapes and are named accordingly. It is customarily given to guests in marriage ceremony. Prior to marriage, the groom has to provide *Lakhamari* to the bride's family which is sent along with the invitation. *Lakhamari* can be stored for several days.

Lakhamari is prepared from rice and black gram flour. Thick dough of the flour is made by adding water. It is then taken in hand and moulded to make different kinds of shape and deep fried in oil. It is then dipped in sugar syrup for 2 h. *Lakhamari* is ready after draining the syrup.

Ayanthamari is prepared by *Maida* and *Math* is prepared by *Anta*. Preparation method and cunsumption pattern of *Ayanthamari* and *Math* is similar to *Lakhamari*.

Paun Kwa

It is prepared by mixing spices, salt, *Hing* and *Lapsi* powder, mixed to water. The slurry is boiled. The soup resulted is known as *Paun Kwa*. It has sour taste.

Sisa busa

Sisa busa is a product similar to salad. Pieces of cleaned raw vegetables like Cucumber and Radish are mixed with the soaked small pea. Little salt is added. Now *Sisa busa* is ready to serve. As soon as this dish is served, it means feast is over.

Concluding remarks

Nepal is a country where around 90 % of people are engaged in agriculture. Varieties of traditional fermented and non fermented foods and beverages are consumed in *Newari* community. Traditional foods and beverages have great significance in festivals. *Newars* are fond of traditional foods and beverages. They prepare different fermented and non fermented delicious dishes and beverages using simple methods and locally available agricultural commodities of the region such as cereals, pulses, meat, vegetables etc.

Newars have great cultural heritage. Several preparations are made during community feast. Festivity and food specialty are more or less synonymous in *Newari* community. They are hardcore ethnic group following their culture and customs strictly. Technology of fermented foods and beverages of *Newari* community is secret and passed from mother to daughter or generation to generation.

Traditional foods and beverages have significant role in the economic, social, religious, and nutritional well being of *Newari* community. However, complete scientific informations on various aspects of traditional foods and beverages are still lacking.

Fermented foods and beverages with probiotic characteristics have been proven beneficial to the health of the population. The study of these fermented products on isolation and identification of pure culture and preparation of these products by using pure culture would eliminate pathogenic and toxin producing microorganisms. So that pure and improved quality products can be produced and it will ensure the health benefits of *Newari* community as well as to other community.

Despite the popularity, nutritional significance and importance of traditional foods and beverages of *Newari* community, scientific methods of preparations of these products have not been developed. Similarly, scientific study of sequence of food service, consumption, as well as food safety is yet to be done. It is highly recommended to explore the scientific study of traditional fermented and non fermented foods and beverages of *Newari* community so that safe and nutritious products can be produced and consumed which can be passed to other communities too.

Glossary of Local words

Achar- Pickle
Ari- Large steel bowl
Aunsi- The dark moon day
Ayanthamari- A kind of sweet
Ayella- Alcoholic drinks
Baji- Cheura- Beaten rice
Bakula - Broad Beans
Bayo- A kind of bread
Bhakari- Bamboo container used to store rice and paddy.
Bhegat- Large mud bowl
Bhuttan – Fried meat dish of different organs
Chaku- Solid balls made from concentrated sugar cane juice.
Chatamari- A bread of rice flour
Chhon- Spicy bamboo shoot
Chhoyala- Marinated and roasted meat
Chhyala- Spicy bamboo soup
Chourasi byanjan- Eighty four types of dishes offered to goddesses
Dahi- Yoghurt
Degudeopuja-Puja offering to festival god
Denalapte- Plate made up of sal leaves
Devta - God
Dewali- Festival day
Dona- Bowl made up of sal leaves
Ginghe macha- A kind of small fish
Guthi- Religious trusties
Gway- Bettlenut
Haku musya- Black soyabean
Handi- Round bottomed and short necked mud container used for roasting
Hing – Asfoetida
Hing- Asfoetida
Janaipurnima (or Kwati purne) - Full moon day of August
Jand- A beer type alcoholic beverage
Janto- Stone grinder designed for splitting and grinding
Jwano- Parsley or ommum
Kachila- Marinated and raw minced meat
Keema- Grounded buff meat
Khaya- Butter milk
Khen- Egg
Khoa- Solid product obtained while concentrating milk
Kucho- Broom
Kunya- A kind of medium sized fish
Kunya- A kind of medium sized fish
Kwaghasa- Thick broth from left over food products has distinct smell and taste.
Kwati- A soup prepared by nine types of pulses
Laba- Green garlic
Lago- A kind of meat ball
Laincha ghain - Green vegetable used in Kwati
Lakhamari-A kind of sweet
Lapi- A kind of meat bread
Luci- Wooden pestle
Luciku- Bamboo scraper
Lwang- clove
Mana- Strater culture made from wheat flakes
Manapu- Starter culture made from rice and millet flour
Manawasha- White flower of wild plant as source of yeast.
Masu- Meat
Masyang - Monthbeans
Math- A kind of sweet
Mayo- A kind of bread
Mee- kegu- Curry of fenugreek and small pea
Momo- A kind of bread with meat inside it and steamed
Murcha – Starter culture used for alcoholic fermentation
Nanglo- Bamboo tray
Pala- Large bowl of mud
Panchhare day- A festival day
PaunKwa- Acidic sour soup
Phanka- Karkalo
Poko-A fermented rice product
Prasaad- Offering to god
Puja thali-Utensil plate used to worship god
Pwasya- Alcoholic drink
Raksi- Distillate from fermenting mass

Sagun- Food offered as sagun which confers good luck and protects against evils.
Salincha- Small bowl of mud
Samay baji- Group of dishes offered to god
Sanya- Dried fish
Sanyakhuna- Jellied fish soup
Simi - Fieldbeans
Sisa bhusa- A kind of salad and small pea
Sithi nakha- A festival day
Sukuti- Dried meat
Syabaji-Roasted beaten rice
Takha- Jellied meat
Tawa-Fry pan
Thalthale masu- Jelled meat
Thayabhu- A plate of bronze to offer *Chaurasibyanjan*
Tunka- Mustard leaves
Tusi- Cucumber
Urghal- Wooden container designed for flaking or pressing
Waaunchha – green vegetables
Wo- Bread made up of black grams or green grams.
Yomari- A conch shaped bread

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Common Tea Pests and Use of Pesticides in Tea in Nepal

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A study was conducted to assess common tea pests and pesticides used in tea in Nepal. The study showed that about twenty four types of common insects, fungus and mites are noticed as the major tea insects and pests. During tea cultivation, around twenty five types of pesticides are being applied to control insects and pests. Contamination of pesticides in tea has a major unrecognized risk to public health. Pesticide residues thus occured can have long-term health implications. Tea is one of the most potential exportable agri-products in Nepal. This article highlights about the major tea insects pests and presents the existing practices of pesticides application in tea in Nepal.

Keywords: Tea, Pests, Pesticides, Pesticide Residue, Safety, Nepal

Introduction

The origin of tea as a popular wonder drink is steeped in legends. In 17th century, tea begins to occupy the right place as a popular drink in china (Jhowar, 2002). Lu yu published first tea classic, contain the detail of cultivation and preparation of tea in china in 780 (Hill, 1998) and different types of green, black, and Oolong tea is made their first appearance of Ming dynasty during 1368-1644. In early 8th century green tea was transferred to Japan for medicinal use (Chu *et al.*, 1997). The tea drinking habit gradually spread along the trade routes of Asia and was introduced to Europe by Dutch traders in 17th century. In Nepal, tea cultivation was started in 1863 AD. Late Gajaraj Singh Thapa initiated plantation of tea in *Ilam* district (Ghimire, 1997). At present tea has been extended to other districts where tea cultivation is made at commercial scale (Tea & Coffee, 2000; ABPSD, 2006).

Growths of tea sector in the country have several advantages. It substitutes the import and earns foreign currency, generates employment as it is labor intensive, benefits environment, as it utilizes hilly unproductive land to production of orthodox tea and decrease migration rate from hills to terai. Therefore, its growth develops nations' socio-economic status. Nepalese orthodox tea due to its smell, unique taste and color of the extract is very famous in the international market (Thapa & Shakya, 2006).

During cultivation of tea, many pests may attack it. Tea growers apply several agro-chemicals including pesticides. Pesticides being toxic in nature do not differentiate between target and non-target species and threaten the health and well-being of humans and wildlife in every region of the world (Wassemann & Wassemann, 1972). These highly stable compounds can last for years and decades before breaking down. They are highly toxic, causing an array of adverse effects, notably death, diseases and birth defects among human and animals. Specific effects can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders, and disruption of the immune system (Strecct, 1981; Maroni, 1990).

In Nepal, about 319 types of pesticides (Insecticides-213, Fungicides-71, Herbicides-23, Rodenticides-8, Acaricides-2 and others-2) have been registered for use under Pesticides Act and Rules of Nepal. Highly persistent types (Chlordane, DDT, dieldrin, aldrin, heptachlor, mirex, toksafen, BHC, Lindane), Phosphamidon and Organo-mercury fungicides are banned in Nepal. Large persistent chemical pesticides have been banned for agriculture and public health from 9th April, 2001. Hazardous pesticides was phased out by the government from 9th April, 2001. At present, prohibition on the use of highly toxic pesticides in tea are quinalphos, ethion, monocrotophos and phorate (Palikhe, 2005). This article wishes to explore the available informations about the common tea pests and existing practices of pesticides applications in Tea in Nepal.

Methodology

Three major tea producing districts in the country representing three different agro-climatic zones (*Jhapa*, *Ilam* and *Panchthar* districts which account for more than 95% of the total tea production in the country) were selected for the survey purpose. Three greatest tea producing village development committees (VDC) within these discricts were selected. Each VDC of the district enlisting of largest tea growers were selected. From one VDC, ten growers were interviewed. VDCs selected were *Anarmani, Gaurigunj* and *Garmani* in *Jhapa* district, *Panchakanya*, *Shreeantu* and *Phikkal* in *Ilam* district; and *Phidim*, *Yangnam, Ranitar* in *Panchathar* district. Field visit and observation of tea gardens, interview with structured questionnaire with personnel communication were served as data collection tool. The collected data were further analyzed and assessed with several literatures reviewed.

Results and Discussion

Tea cultivation and processing

Currently, in Nepal 134 tea states and tea gardens were recorded and among them 89 were registered at National Tea and Coffee Development Board (NTCDB) to the year 2006. Tea industries in Nepal mainly falls into three categories i.e.

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the industries which only process tea, tea industries having tea garden as well as processing and tea packaging industries. There are 13 industries in first categories, 28 in second categories and 20 are in third categories. The tea states still remain to register to NTCDB accounts for fourty-six. Among the registered tea states and gardens, 57 are situated on *Jhapa* district, 10 tea states are in *Ilam*, 5 are on *Dhankuta* and 17 in other districts of Nepal. The total land used for tea plantation occupy 16012 hectare and total production is more than 13.68 million kg. The land used for orthodox tea production comprises 7036 hectare and 8976 hectare for CTC tea plantation (NTCDB, 2006). The major CTC tea plantation area represents mainly *Jhapa* district. Orthodox tea plantation and production area are hilly district like *Ilam, Terhathum, Dhankuta*. It is expanding to other hilly area particularly in *Sindhupalchowk* and *Nuwakot*. *Sindhupalchwok, Dolakha, Ramechhap, Sankhuwasabha, Bhojpur, Gorkha, Kaski* and *Solokhumbu* are the districts where the tea plantation was recently started. Due to the lack of financial adequacy and effective policy implementation for small producers, tea cultivation has not been very popular at the farmer's level. (Thapa, 2005)

Black tea is mostly consumed in Nepal. Nepal's specific geographic, agro-climatic and environment friendly agriculture system has favored to capture international market. All together 7154 small farmers are engaged in tea cultivation. There is growing international and domestic market of tea that is why average growth rate of tea in the country is 17 percent per annum. Tea industry purchase green tea leaves as a raw material from the tea growers and process it. Very few have their own tea garden that is insufficient to fulfill the plant capacity which makes them to additional collection from other growers. The technology of cultivation of tea in the country transferred from *Darjeeling* and *Assam*, India. Mostly tea technician working in this industry has been trained in India. Due to the suggestion of Indian technicians and pesticide dealers with open Indian boarder there is uncontrolled use of pesticides in tea during its cultivation.

Tea could be one of the valuable cash crops in Nepalese agro economy, but the pesticides residue in tea become one of the biggest and touching problem on export of Nepalese tea. Nepalese tea encountered with the problem of pesticides time after time and several consignments have been also rejected by the importing country. Effective tea development Policy along with scientific agriculture practice, efficient quality control mechanism and technolgy for cultivation, harvesting production, processing, storage and distribution is current need to strengthen Nepalese tea economy and consumers' safety.

Common Tea Pests and Use of Pesticides

The result showed that different types of pesticides are used in tea cultivation to control the varieties of insects and pests. The use of organophosphate includes quinalphos, ethion, malathion, monochrotophus, propanofus, acefate, dichlorovous, chloropyriphos were found in the tea field to control large verities of pests like caterpillar (*Latoia spp., Andraca bipunctata, Andraca bipunctata, Gracilaria theivora*), leaf roller (*Gracilaria theivora, Stringlina glareola, Hamona coffearia*), tea mosquito (*Heolipeltis febriculosa*), aphids (*Toxopetra aurantii, Empoasca flavescens*), jassids (*halopeltis spp.*), flush worm (*Lasperesia bipunctata*) and other pests. Similarly synthetic insecticides such as alfamethryene, cyper methryene and pyrethroid ester insecticides like fenpropathrin were also used in tea. Herbicides such as gramoxone, oxyflurene, glyphosphate and 2, 4 D were generally found to use to control dicot, broad leafs and grass bushes.

Fungicides like copper oxychlorides, hexachonazol, carbandazim, mancozep, and copper sulphate were used in tea cultivation to prevent from fungal disease and foliar disease such as red rust (*C. paraciticus*), black rot (*Cephaleuros mycoldea*), and blister blight (*Corticulum invisum*). To control and prevent the different mites problem during tea cultivation, accaricides such as dicofol, prapargite, sulphur and fenopthrin were used .The survey also revealed that use of prohibited pesticides in tea i.e.quinalphos, ethion, monocrotophos were still in use. Some of the Pesticide (dichlorovos) used in tea are under restricted pesticides which should be used under approval of Plant Protection Officer. Among these pesticides, dichlorovos and monocrotophos are included in highly hazardous group (Ib) of pesticides by WHO. Common insects and pests observed in tea is given in Table 1 and the major pesticides used in tea is presented in Table 2.

Table 1: Common insects and pests in tea in Nepal

Common name of insects & pests	*Scientific name*
INSECTS	
Thrips	*Andraca bipuntata*
	Scirtothrips dorsalis
	Hallothrips andressi
	H tenipennis
Slug caterpillars	*Latoia sp*
Bunch caterpillars	*Andraca bipunctata*
Caterpillar	*Andraca bipunctata*
Leaf roller	*Gracilaria theivora*
	Stringlina glareola
	Hamona coffearia
Looper	*Biston suppressaria*
Tea mosquito	*Heolipeltis febriculosa*
Aphid	*Toxopetra aurantii*
	Empoasca flavescens
Jassids	*Helopeltis spp*
Halopeltis	*Lasperesia bipunctata*
Flushworm	*Lasperesia bipunctata*
FUNGUS	
Blister blight	*Corticulum invisum*
Black rot	*Cephaleuros mycoldea*
Red rust	*C. paraciticus*
MITES	
Pink, purple and red mites	*Tetranychus biocuatus*
	A theae
	Acerina gosspii
	Oligonychus coffeae

Table 2: Pesticides applied to tea cultivation in Nepal

Trade Name of pesticide	Common name of the Pesticides	Formulation /Group	Frequency used	Month	Sensitive pests
Thiodan	Endosulfan	Chlorinated Acaricides, Cychlodiene Insecticides	2	January, February	Green Fly, Looper, Red slugs, Thrips Aphids, Flushwormes etc.
Farsa, Gem	Alphamethryene	Synthetic parathroids	2	April, September	Tea Mosquito, Thrips,
Flash , kinalaux	**Quinal phos	Organophosphate acaricides,Quinoxalline organothiophosphate insecticides	3	March,Aug,	Jassids, Aphids Broad spectrum Insect
Emite	**Ethion	Organophosphate acaricides,Aliphatic Organothiophosphate insecticides	4	March-August	Catterpillar , thrips, Red spider, pink .scarlet and purple mite
Monosil	**Monocrotophos	Organophosphates	2	April, October	Green Fly, halopaltis, Thips, Looper, Jasids, Caterpillar
Malathion 5% DP	Malathion	Organophosphate Acaricides/Aliphatic Organothiophosphate	2	March, April	Tea mosquito
Propafos	Propanophos	Organophosphates	1 or 2	as necessary	Thrips,Aphides, worms etc.
Emidagold, Josh	imidacloprid	Chloro-nicotinyl	1 or 2	as necessary	Termites and other insect
Orthene, Acecap	Acefate	Organophosphates	1 or 2	as necessary	Chewing and Sucking pest
Ripcord	Cypermethrine	Synthetic Parathroids	1 or 2	as necessary	Wide range of pest.
Nuvan 76%	***Dichlorovos	Organophosphate	1 or 2	as necessary	Tea mosquito, Jassids, Fly larvae
Durmet	Chloropyriphos	organophosphate	1 or 2	as necessary	Mosquitos,fly larvae, Aphids other insect and termites
Gramoxone	Gramoxone	Herbicides	1	July Aug	Dicot /Broad leafs,
Round up	Glyphosphate	Organophosphorus Herbicides	1	March/April	Pre-emergence of Dicot /Broad Leafs
Goal	Oxyfluren	Diphenylether Herbicides	2	Sep, March	Prevent post emergence of weeds (used in Young Tea)
2.4 D	2,4 D	Phenoxy Acetic Herbicide	1or 2	as necessary	Broadleaf weeds
Blitox	Copper oxychlorides	Oxychloride	3	Feb,Apr, June	Red Rust
Contaf	Hexachonazol	Conazol	1or 2	as necessary	Fungas Infestation
*Na	Carbandazim	Benzimidazole	1 or 2	as necessary	Fungus disease and infestation
Dithane, Mancopius, PenncoZeb	Mancozep	Polymeric dithiocarbamate	1 or 2	as necessary	Wide range of Foliage disease
Bordeaux	Cupper sulphate	Copper fungicide	1 or 2	as necessary	Fungus disease and infestation
Dicofol18.5%EC	Dicofol	Bridged diphenyl	2	Mar. April	Mite
Omite	Prapargite	Other Insecticides	2	Mar, April	Mites, spider
sulphex	Sulfur	Inorganic	1	March	Red spider, other mites
Dennitol	Fenpropathrin	Pyrethroid Ester	3	Marh- May	Mites

** Prohibited Pesticides in Tea

*** To be sold and use under official recommendation of Plant Protection Officer.

Assessment of pesticide residues

Some of the preliminary assessment on the pesticide residues in tea in Nepal has been carried out. The analysis result from 1995 to 2004 showed that out of 74 samples of tea, 22 samples (29.7%) were positive to pesticides contamination. Among the pesticides detected samples, 12.5% samples showed presence of BHC (range: trace-1.4 ppm) followed by 8.3 % samples showed malathion (range: 0.2-10 ppm), 2.7 % samples showed the presence of parathion and methyl parathion (range: 0.2-2.5 ppm) and 1.35 % samples showed the presence of DDT (range: trace-0.2 ppm).The residue of banned pesticides like DDT, BHC appeared in final processed tea (Table 3).

Table 3: Compiled laboratory data of Pesticides residue in tea (from 1994-2004) in Nepal

No of samples	*DDT*		*BHC*		*Malathion*		*Parathion*		*Methyl Parathion*	
	Detected	*Range*	*Detected*	*Range*	*Detected*	*Range*	*Detected*	*Range*	*Detected*	*Range*
74	1	+Tr-0.2	9	+Tr-1.4	6	0.2-10	3	0.2-2.5	3	0.2-2.5

+Tr =Trace

(Source: Annual Bulletin, DFTQC, 1994-2004)

Export Import Scenario

Total tea production in the year 2005/06 is 13.68 million kg, of which orthodox tea production covers more than 1.65 million kg and CTC tea covers 12.03 million kg. Among them more than 95 % orthodox tea was exported while the CTC tea market is limited to domestic consumption only.Nepalese tea export has been increased from the year from 2000 to 2005 but in 2006 it was decreased by 80.67 % in the comparison of previous year. The tea consumption rate is 0.35 kg per person per year in Nepal (NTCDB, 2006).The status of tea plantation area, production and export of Nepalese tea is summarized in Table 4.

The major tea trading partner is India where more than 1100 tons of tea was exported in 2005 whereas 97 tons of was exported to third countries and local consumption was 25 ton.The international market for Nepalese CTC tea is limited to India and Pakistan only while the international demand to Nepalese orthodox tea has been expanded to Japan, Germany, US and even to European nation due to its health benefits and good quality. On other hand continuous efforts of private sector engaged to promote Nepalese tea in international market particularly in Europe, Japan and boosting up the cultivators and processor to acquire organic certification. Introduced tea policy in 2000 is expected to encourage the tea industry in Nepal.

Table 4: Plantation area, production and export status of Nepalese tea

Year	*Plantation area in hectare*	*Production in metric ton*	*Export in metric ton*
2000/1	1197	6638.08	69.5
2001/2	12346	7518.58	79.6
2002/3	12643	8198.00	193
2003/4	15012	11651.20	984
2004/5	15900	12606.08	4316
2005/6	16012	13688.24	834

(Source: NTCDB, 2006)

Concluding Remarks

Tea is a one of the major cash crops in Nepal. Nepalese orthodox tea has an increasing demand in international market Tea export from Nepal has been reduced in current years due to pesticide residue and other inorganic residue that comes from agricultural practices such as use of pesticides and chemical fertilizers. Nepalese tea will not be able to utilize the opportunity of WTO if it contains banned and prohibited pesticides and the level of pesticide residues beyond the specified level.

Plant protection Directorate is being responsible for implementing pesticide act in the country. Food law is silent in pesticide MRLs in tea. There is lack of sophisticated laboratory in the country capable of detecting different pesticides. Stakeholders for the promotion of tea sector in Nepal including, Plant Protection Directorate, Nepal Agriculture Research Council, Tea Coffee Development Board, Tea association, Farmers association should unite together in advocacy campaign for pesticide law enforcement, for discouraging the use of chemical pesticides and for the promotion of alternative approach such as IPM, organic farming and the use of bio-pesticides. So, the strict quality assurance measures such as GAP and HACCP should be implementated in the entire food chain i.e. from farm to fork. Use of organic fertilizers, promotion of the IPM approach and the use of safe and bio pesticides are essential. This may help to facilitate tea trade in the international market as well as to safeguard the consumers health in the country.

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Hazard Analysis and Critical Control Point (HACCP) in Pig Slaughtering in Nepal

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The Hazard analysis and critical control point (HACCP) system is an internationally agreed approach to food safety control which can be applied to a wide range of simple and complex operations. The condition of animal handling, meat production and marketing in Nepal is still primitive. Slaughterhouse and Meat Inspection Act of Nepal has been made with the aim to control haphazard system of production and marketing of meat. Compliance with HACCP requirements or third party certification, will safe guard the safety of meat and leads to higher satisfaction and lesser complaints from customer. Similarly, adoption of HACCP will increase the export potential of meat and meat products. This article highlights the current slaughtering practices and HACCP approach for safe pig meat production in Nepal.

Keywords: Hazards, Critical control points, Hygienic principle, Infection, Zoonotic disease, Slaughtering

Introduction

Maintaining food safety is a complex and multifaceted challenge requiring all who produce, process, distribute and sell food products to play an increasingly proactive role. Demand for safer food is growing as consumers become more affluent, live longer, and better understand the links between diet and health (Woteki *et al.*, 2004). Food hazards introduced accidentally or deliberately can be classified as physical, chemical or biological (Escriche *et al.*, 2007). Microbial agents that are widespread can lead to significant hazards and are often difficult to detect, and can re-enter the food supply chain even after control at earlier stages. The issues are more important in case of Meat item either during pre-slaughtering condition of meat animal, slaughtering or post slaughter treatment and processing of meat. Meat is an ideal culture medium for many organisms because it's high moisture, favorable pH, nitrogenous compounds, minerals and accessory growth factors, and fermentable carbohydrate (glycogen) (Frazier & Westhoff, 1997). Thus, meat and meat animal can be the source of zoonotic diseases, pathogenic micro organisms and potent toxins, that are harmful and some time lethal to humans (FAO, 1991). Approximately one-third of global meat exports (6 million tones) are affected by animal disease outbreaks (Anon., 2004).

Livestock currently sustain the livelihoods of an estimated 700 million rural poor in developing countries. In Nepal, poverty levels are extremely high and more than 80% of the population relies on the agriculture sector for employment and income generation (Maltsoglou & Taniguchi, 2004). The contribution of livestock to national GDP is almost 11 % and 70 % of household keep some types of livestock including cows, buffaloes, pigs and chickens. Regarding the number, pigs occupy only 2.4 % of total livestock (37 million), which is in fourth position after poultry, buff/beef and sheep/goat. The annual production of pig meat is 14.38 thousand ton (7.40 %) out of 0.2 million metric ton of total meat production (Pariyar, 2005). This refers to 9.2 kg meat consumption per capita, which is higher than 1.1 kg reported by FAO in 1991. The annual growth rate of pigs in number and quantity of meat are around 4.5 % which is higher than buff/beef, and goat/sheep. It is concluding that the consumption of pig meat is increasing trends where as other excluding chicken are in decreasing trends. The common breeds of pigs in Nepal are *Hurrah, Chwanche, Nagpuri, Hampshire, LargeWhite Yorkshire and Pakhribas pig* (Joshi & Olesen, 1999). Delgado *et al.* (2003) predict a further near doubling of the demand for meat in the developing world, with the result that the developing countries in 2020 would out-demand the developed world in meat.

Annual consumption of meat in developed countries in year 2002 was 76 Kg meat per person per year, compared to 25 kg person per year in developing countries. Pork meat occupies first position on both worlds with 38 % and 48 % respectively (FAOSTAT, 2005). Due to case of BSE and avian flue, FAO expects that demands for substitutes such as pork to increase significantly (Anon., 2004).

The condition of animal handling, meat production and marketing in Nepal is primitive. Pre-slaughter handling is cruel, ante mortem and postmortem inspections are not yet practiced. The slaughtering condition is far from satisfactory, along with filthy and totally unhygienic transportation and meat marketing. The meat is often adulterated with poor unhygienic meat or meat from different species. Environmental pollution by indiscriminate disposal of inedible by-products, rumen content, condemned meat, and effluents is a serious problem. Thus, the consumers are deprived from clean and wholesome meat in spite of their fundamental right for it (Joshi & Olesen, 1999; Subba, 2002; TLDP, 2003). Though the meat consumption is partially governed by races and religion; the growth of tourism, increase in income of urban people, and

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change in consumer food habits towards meat item have created a steady demand for quality meats. Consumer complains about unavailability of better cuts, inferior marketing practices and lack of proper hygiene are often seen as headlines news these days. In response to these demands, few organized sector are beginning to emerge which are more selective with their livestock purpose and are adopting better standards of hygiene (Joshi & Olesen, 1999). However, established big slaughter house e.g. Everest Foods Ltd (Hetauda) are unable to operate due to lack of awareness on consumers regarding meat safety and weak legislation inside country (Subba, 2002; TLDP, 2003).

Slaughter House and Meat Inspection Act (1998) and Rules (2000), administered by Department of Livestock Services with in the Ministry of Agriculture and Cooperative, have been enacted with the aim to control with a view to protecting public health from adulteration of meat and other activities that affect the meat quality. The act requires slaughtering at approved premises, humane slaughtering, inspection of live animal before slaughter, examination of meat (carcass) for zoonotic disease, and maintenance of basic hygienic codes. The provision has been made to obtain license for operation of slaughter house, meat inspector for inspection and maintenance of quality of meat and livestock, meat supervisor to supervise the activity of inspectors etc. Penalties under the act consists of fines ranging from NRS 5000 for a first time violator to a NRS 10000 fine, one month's imprisonment or both to a second time or subsequent violator. As meat fall under the definition of 'foodstuffs' under the Food Act (1966) and Regulations (1970), administered by Department of Food Technology and Quality Control, these legislations have prohibited production, sale and export of the meat that is adulterated, spoilt or produced from unhealthy animals. No quality specification meat cut parts and products are yet formulated .To safe guard the public health and environment, and for effectiveness of meat trade, effective implementation of regulations and act and formulations of quality specification of meat and meat product is the need of time (Subba, 2002). Animal Health and Livestock Services Act (1998) and Rules (2000) has been enacted with the view to developing animal husbandry including the healthy production , sale, distribution, export and imports of animals, animal products or animal product inputs. Acts provides the establishment of quarantine check posts and rule has made detail provisions on quarantine, procedures and quarantine certificates. Similarly, Local Self–Governance Act (1999) and Rules (2000) empowered Municipalities to arrange for the establishment and monitoring of animal slaughter house. In spite of these legislative measures, animal slaughtering and meat marketing with in country is far from satisfactory, illustrating the nonfunctioning of legislative measures.

Pig production is concentrated in hilly region because of resident ethnic groups such as *Rais, Limbus, Magars, Tamangs*, and *Tharues*. However other castes such as *Brahmin*, *Chetries*, *Tamangs,* and *Gurungs* have also started raising pigs (TDLP, 2003). A survey on 1999 showed that in Kathmandu valley, pigs are slaughtered regularly in 18 places, most of them near the river bank. The other places include country yard, old shed, bushy areas or any where convenient like open places. The carcass or cut parts are transported to shops manually or on bicycle, rickshaws, cart or motor vehicle. Pig wholesale cuts are hanged all over the day for selling purpose (Joshi & Olesen, 1999). The slaughtering practices of pig in most part of country follow the same methodologies. During the transportation, meat animal are confined at very small areas with no provision of drinking, feeding and resting. During transport and in lairage, mixing of unfamiliar pigs are common, which cause fighting and social stress. Pigs are sometime tied up by rope and carried on the cart. None of the animals are subjected to ante mortem examination, however diseased and wasted animal are priorities for slaughtering. Pigs are left on the field and are killed by inserting a long javelin forcefully on the heart area. The insertion is carried out by an experienced person. The pig, thus forcefully fall on the ground. No attempt is done to collect the blood released after insertion. Once the movement of the pig is seized, the animal is carried to the slaughtering yard (any area with hard surface), where the hair is removed buy moving torched flame over the body using kerosene. The burnt hair is removed with blunt knife as scrapper. The minor hair particles are shaved by razors using water. A superficial cut is done between two hams, cut is continued towards pizzle and skin is cut dip around the preputial pouch. The pizzle is freed and small opening is made through the abdominal wall. The connective tissue tying the bungs is cut and bungs are removed. In the mean time the internal blood are collected. The pluck (liver, heart and lungs) are removed. The carcass is split to two halves from middle, one part consisting of ham part and other of foreleg parts. The whole sale cuts are made and send to meat shop scattered very where in town and city. In rural areas, and majority part of *terai* regions, no specific shops are opened but are sold on weekly markets called *Haat Bazar*. No use water is done except on the shaving of skin (Personal observation, 2007). Thus, the faulty transportation of meat to slaughter area, lack of postmortem and ante mortem inspection, unhygienic slaughtering process, inappropriate handling of meat and its byproducts, lack of maintenance of cold chain, avoidance of basic hygienic principle for safe food, make pig meat an unsafe food to consume by general consumer.

Microbiology of pig meat

Meat is an ideal growth medium for many organisms due of favorable water activity, pH, nutrients and growth factors (Frazier & Westhoff, 1997). Muscle tissue of healthy animal is, in principle, free from micro organisms. Bacterial contamination of carcasses may occur at virtually every stage of slaughtering and processing. The sources of contamination may include contaminated equipments (cutting tools,

chopping blocks, containers etc), the surrounding air, water and carrying agency. Man, as a carrier of different organisms, is the most important factor in the area of secondary contamination (Heinze & KGaA, 1987). Although this contamination seems, to a certain extent, to be inevitable, the level can be increased or decreased substantially by bad or good slaughter procedures. Processing hygiene, however, aims at holding the initial bacterial numbers on a level as low as possible, since this affects shelf-life and the occurrence of pathogenic bacteria on meat (Upmann *et al.*, 2000).

The essential problem in many developing countries is the failure to provide for hoists or hooks that permits the dressing of carcasses to take place off the floor. The contamination resulting from floor dressing of carcasses is considerable, especially where the removal of hides and the cleaning of stomachs are carried out in the same location as the dressing of the carcass itself. Personal hygiene and particularly keeping the hands clean are important in relation to the spread of *Salmonella* of pathogenic varieties (Heinze & KGaA, 1987). De Wit & Kampelmacher (1981) found that in slaughterhouses, for instance, the hands of personnel were contaminated 65 to 100 % with *Staphylococcus aureus*, a bacterium that causes meat poisoning, 86 to 100 % with the faecal organism *Escherichia coli* and faecal Streptococci and 5 to 36 % with *Salmonellae*. Personal hygiene and particularly keeping the hands clean are therefore increasingly important in relation to the spread of these microorganisms. Holding animals in vehicles or lairage without adequate drainage of litter, frequently results in fecal soiling of the skin. In these cases, the knife will have to cut through manure and fecal residues, resulting in a great possibility for meat contamination by faecal pathogens (Chambers & Grandin, 2001).

Pork meat may be contaminated with a range of human pathogens, including *Salmonella*, *Campylobacter, Escherichia coli*, *Yersinia* and *Listeria, Staphylococus aureus*, *Clostridium botulinum* and *Clostridium perfringens.* These microorganism may come from hide, soil adhering thereto, the content of gastro intestinal tract (if inadvertently released during dressing operation), airborne contamination, aqueous source (water used for washing, scalding, cleaning of carcass; and cleaning the floors), the instruments used for dressing (knives, saws, cleavers, and hooks), various vessels and receptacles, and finally the personnel) (Lawrie, 1979; Mafu *et al.*, 1989; Hald *et al.*, 1999; Bolton *et al.*, 2002). Consequent cross-contamination of the meat thus becomes inevitable. The consumption of such, at risk, products obviously contributes to the overall burden of food borne disease in the wider community. It is very difficult to derive precise correlations between pathogen incidence in foods such as pork and rates of clinical report, however, some data are available. For example, the estimated average incidence of salmonellosis in the Netherlands is about 450 per hundred thousand of the population, with an estimated 15% of these being associated with pork consumption (Berends *et al.*, 1997). No such epidemiological data are found in our country (Subba, 2002). In spite of these external bacterial contaminations, various parasites are found with in or transmitted by skeletal muscle. Pig meat may contain *Sacrocystis*, *Trichinella spiralis*, *Hydatidosis* capable of infecting humans. Pork intramuscular connective tissue may horbour infectious *Taenia saginata* and *Taenia solium*. Similarly, meat animal might be infected with various diseases which are transmitted to other healthy animals and humans from direct contact or from meat. Anthrax, Brucellosis, Rabies, Histoplamosis, Tuberculosis are some common zoonotic diseases (Lechowich, 1971; Subba, 2002; Chaudhary *et al.*, 2006).

Hazard analysis and critical control point

Food safety is an increasingly important public health issue. The hazard analysis and critical control point system (HACCP) is the internationally agreed approach to food safety control. The reference standard for implementation of HACCP is published by the Codex Alimentarius Commission of the joint United Nations Food and Agriculture Organization (FAO/ WHO) Food Standards Program. Similarly, ISO 22000:2005, Food Safety Management System – Requirement for any Organization in Food Chain, has integrated HACCP on its requirement standard (Codex, 2004; ISO, 2005). HACCP is designed to control significant food safety hazards, i.e. those hazards that are likely to cause an adverse health effect when products are consumed. In order for HACCP to be effective, it is essential that it is supported by Good Manufacturing Practice (GMP) (Sperber,1998); Sanitation Standard Operating Procedures (SSOPs) (NACMCF, 1999) or prerequisite programmes such as Good Hygienic Practices (GHP), appropriate codex code of practice and appropriate food safety requirements (Codex, 2004). Consistent maintenance of these programs is important to the success of the HACCP plan. GMP'S emphasize sanitation effectiveness and hygienic practices which should occur during food processing. An effective GMP program will help reduce the level of spoilage and pathogenic microorganisms. SSOPs are widely used program to maintain proper sanitation within food processing plants. They describe all daily procedures that will be conducted to maintain sanitation, specify the frequency of the procedures, and identify those responsible for implementing and monitoring the SSOP (Eisel *et al.*, 1997). These practice brings with it the higher costs involved in obtaining the appropriate equipments and paying for the greater length of time required. But these costs are soon recouped by the better quality and shelf life of the products (Untermann, 1990). Snijders (1988) has stated the following elements of GMP in slaughter lines. Al these elements should get sufficient attention. If they do not, it will be impossible to achieve an optimum hygienic end product with a low contamination level.

1. Separation of healthy and sick animals and all measures to prevent animals in general forms acting

as an important source of contamination of the vehicles and slaughter line

2. Hygienic measures during transport
3. Slaughtering in properly hygienic way. In order to achieve this it is necessary to divide the slaughter process into different parts, resulting in a minimalization of cross- contamination. Slaughter lines can be divided into three separate parts:
   - The lairage and stunning area
   - The so-called "unclean part". This comprises all slaughter actions from bleeding to skinning. In pig slaughter line this includes: bleeding, scalding, dehairing, singeing, balckscraping and polishing
   - The so-called "clean part", where evisceration is carried out and the carcass and its organs are prepared for meat inspection
4. Adequate chilling and maintaining the "cold chain" during cutting and transport
5. Optimal cleaning and disinfection supported by bacteriological monitoring
6. Training and instruction of the slaughter line personnel, which includes motivation in order to make them apply the instruction from conviction and not only because they are ordered to do so
7. Good hygiene control during the slaughter process

Some of these elements are found in accordance to the requirement stated in Slaughter House and Meat Inspection Act (1999) and Rule (2001) of Nepal. The act, further specified provisions of antimortem and postmortem inspection by meat inspector or supervisor; separate area for suspects meat and meat animal; separate stunning, bleeding, skinning and viscera cleaning area; provision of hot and cold water; appropriate design of building and its floor, wall ceilings ; water drainage; ventilation; latrine; change rooms etc to maintain the hygiene and sanitation during animal slaughtering. The rule states for appropriate disposal of the solid waste like inedible offals, dirty blood, rumen content, dung litter, inedible viscera and condemned carcass within eight hours of slaughter. Similarly, provision are made for health check up of workmen bi-yearly and no meat are dispatched from the slaughter house with out appropriate tagging from meat inspector.

The Current Good Manufacturing Practice (cGMP) for manufacturing, packing, or holding human food published by FDA can be used for reference GMP in meat industries. Similarly, Codex, CAC/GL 52-2003 and FAO (1991) has published general principles of meat hygiene and guidelines for slaughtering meat, cutting and further processing. These reference materials provide ample guidelines for general hygienic rules for facilities, equipment and personnel in the meat industries.

A key feature of HACCP concept is that by establishing targets or standards, innovation and changes will be stimulated to reduce the risk from all sources of food borne hazards-biological, chemical, and physical-while simultaneously providing a tool for holding establishments accountable for achieving acceptable levels of food-safety performance. There will never be a process that is absolutely safe, but there must always be a constant effort to achieve zero defects (Snyder, 1991; Hulebak & Schlosser, 2002). Another feature of HACCP is to reduce or even eliminate the need for endpoint testing. This testing can be very tedious and time consuming. Also, testing can lead to a loss of product, since some types of testing are destructive. HACCP attempts to reduce endpoint testing by conducting a series of checks through the process. At each step in the process, all possible hazards are considered in regard to prevention and to determine what actions will be taken if a significant hazard occurs. By the time, the product reaches the end of the process, HACCP attempts to reduce hazards to an acceptable level. Another purpose of HACCP is to provide documentation to prove that the process is being conducted as written (Bauman, 1990; Mortimore & Wallace, 2000). The compliance to HACCP can be volunteer activity for internal performance important of organization or used for third party certification as safety assurance measures (Dhital, 2000).

Originally the HACCP protocol consisted of only three principles viz. (1) Hazard analysis and risk assessment, (2) determination of critical control points (CCPs), and (3) monitoring of CCPs. In 1989, the National Advisory Committee on Microbiological Criteria for Foods (NACMCF) added four more principles to the HACCP system (Sperber, 1991). According to the NACMCF (1992) and Codex (2004) and the HACCP system consists of seven principles that outline the establishment, implementation and maintenance of the plan viz:

1. Conduct a hazard analysis
2. Determine the CCPs
3. Establish critical limit(s)
4. Establish a system to monitor control of the CCP
5. Establish the corrective action to be taken when monitoring indicates that a particular CCP is not under control.
6. Establish procedures for verification to confirm that the HACCP system is working effectively.
7. Establish documentation concerning all procedures and records appropriate to these principles and their application.

The application of HACCP principles consists of the following tasks as identified in the logic sequence for application of HACCP (Codex, 2004).

1. Assemble HACCP team
2. Describe product
3. Identify intended use
4. Construct flow diagram

5. On-site confirmation of flow diagram
6. List all potential hazards associated with each step, conduct a hazard analysis, and consider any measures to control identified hazards (principle 1)
7. Determine CCPs (principle 2)
8. Establish critical limits for each CCP (principle 3)
9. Establish a monitoring system for each CCP (principle 4)
10. Establish corrective actions (principle 5)
11. Establish verification procedures (principle 6)
12. Establish documentation and record keeping (principle 7)

From the recommendations and promotional activities of many international organizations, such as Codex, FAO-WHO, FDA, NACMS, UNESCO, and also from the proven benefits of HACCP system, many governments mandated use of the HACCP in their food industries and food business (Escriche *et al.*, 2007). In the United States, HACCP was mandated for seafood in 1994 (Procedures for the safe and sanitary processing and importing of fish and fishery products: final rule, docket no. 93N-0195 1995), for meat and poultry in 1996 (Pathogen reduction HACCP systems: final rule, docket no. 93-016F 1996), and for fresh fruit juice in 2001 (HACCP: procedures for the safe and sanitary processing and improving of juice: final rule 2001); and regulations taking a risk-based approach have been mandated for shell-egg handling (Food labeling, safe handling statements, labeling of shell eggs; refrigeration of shell eggs held for retail distribution: final rule 2000).The European Union directive 93/43, effective in December 1995, requires member states to adopt a HACCP approach in obliging food companies to follow HACCP principles in their production process (Grijspaardt-Vink,1995). The companies, themselves, are responsible for monitoring their food safety, although final authority lies with the national authorities (Bunte, 1999). Australia, New Zealand, and Canada also have mandatory or voluntary public programmes to encourage adoption of HACCP system (Peters, 1997; Dean, 1990). As an outcome of its use in most developed countries, HACCP is increasingly practiced in less developed countries that export food products into industrialized markets (Merican, 1996). As Codex standards, guidelines and recommendations have been identified as international standards under the Agreement on Sanitary and Phytosanitary Measures agreed at the Uruguay Round of GATT negotiations (Karki, 2002), compliance of HACCP will facilitate the international trade of food stuffs (Escriche *et al.*, 2007).

Proposed HACCP approaches for pig slaughtering

Proposed HACCP team

The human resources on the proposed the slaughter house are Food Technologist (1 no) as General Manager (GM), Intermediate veterinary technician (2 nos), operators (mechanical, electrical and intermediate food technician), workers (7 nos). A HACCP team consisting of GM, a veterinary technician, mechanical operator, intermediate food technician and two workers are proposed.

Proposed product description

The proposed definition of meat according to Slaughter House and Meat Inspection Act (1999) and Rule (2001), is "Meat of healthy pig which is fit for consumption". The purposed intended use is consumption by customer after cooking or processing in to different products applicable for all age. The proposed product description is given on Table 1.

Table 1: Product Description of Pig Meat

Product	*Pig meat*
Intended end use	export and domestic wholesale and retail markets as fresh product intended to be cooked
Preservation method	chilling and packaging
Packaging	vacuum packaged (oxygen impermeable plastic bags), packed in new cardboard cartons
Identification and labeling	full trade description
Control during storage	time/temperature to limit microbial growth during chilling process
Control during distribution	time/temperature to limit microbial growth during chilling process
Final customer preparation	intended to be cooked

Proposed slaughtering method for pig

Considering a model slaughter house processing locally sourced pigs at a rate of approximately 50 pigs per day, the following methods are proposed for slaughtering of animal. Upon arrival in lairage, pigs are immediately power hosed with potable water at approximately 19°C, applied at a pressure of approx. 1030 kPa. Animals are then transferred into the abattoir, inspected, stunned using low voltage (60 -80 V) electrical stunning tongs and secured to an overhead conveyor rail by a chain looped around one of the hind legs. The stunned animals are immediately bled by cutting the main blood vessels using a knife. Each animal is scalded and dehaired in a combined process involving individual immersion for 2–3 min in a scald tank (held at temperatures between 60 and 62°C) and dehairing manually using specially formed scrapper (bell scrapper or knife). After dehairing, pigs are secured to an overhead conveyor belt by hooking the

hind legs, toe nails are removed and manually singed using a hand-held gas singer. After singeing, black deposits and singed hair are scrapped off and the animals are given a pre-evisceration wash by power-hosing with potable warm water (40°C) applied at a pressure of approximately 1030 KPa. Evisceration involved three separate tasks (debunging, slitting the belly open and gut removal), all of which are performed by the same operative. Debunging or detachment of the rectum is initially completed before the belly is slit open and the connective tissues joining the bung and viscera to the carcasses are cut. The diaphragm, heart, lungs, trachea and tongue (with tonsils) are manually removed together as part of the pluck set, along with the digestive tract. The kidneys are not removed at this stage. The carcasses are subsequently manually cut along the midline from the hind to the fore using a splitting saw. The heads are not removed, but the spinal cord is cut away. Carcasses receive a final trim after spray-washed with cold (15:°C) potable water to remove bone dust and blood clots before chilling and cold storage. During cooling, the refrigeration system is maintained such that deep muscle temperature is lowered to 6-7°C with in 12-16 h. The chilled meat are graded and packed and finally stored at chilled temperature before dispatch. The carcass is transported on the refrigerated vehicle maintaining the good hygienic practice during dispatch to the consumer or seller. The condemned material is disposed properly and fundamental effluent treatment is done to minimize the environmental pollution from operation. The proposed processing steps and flowchart for pig slaughtering is given in Fig 1.

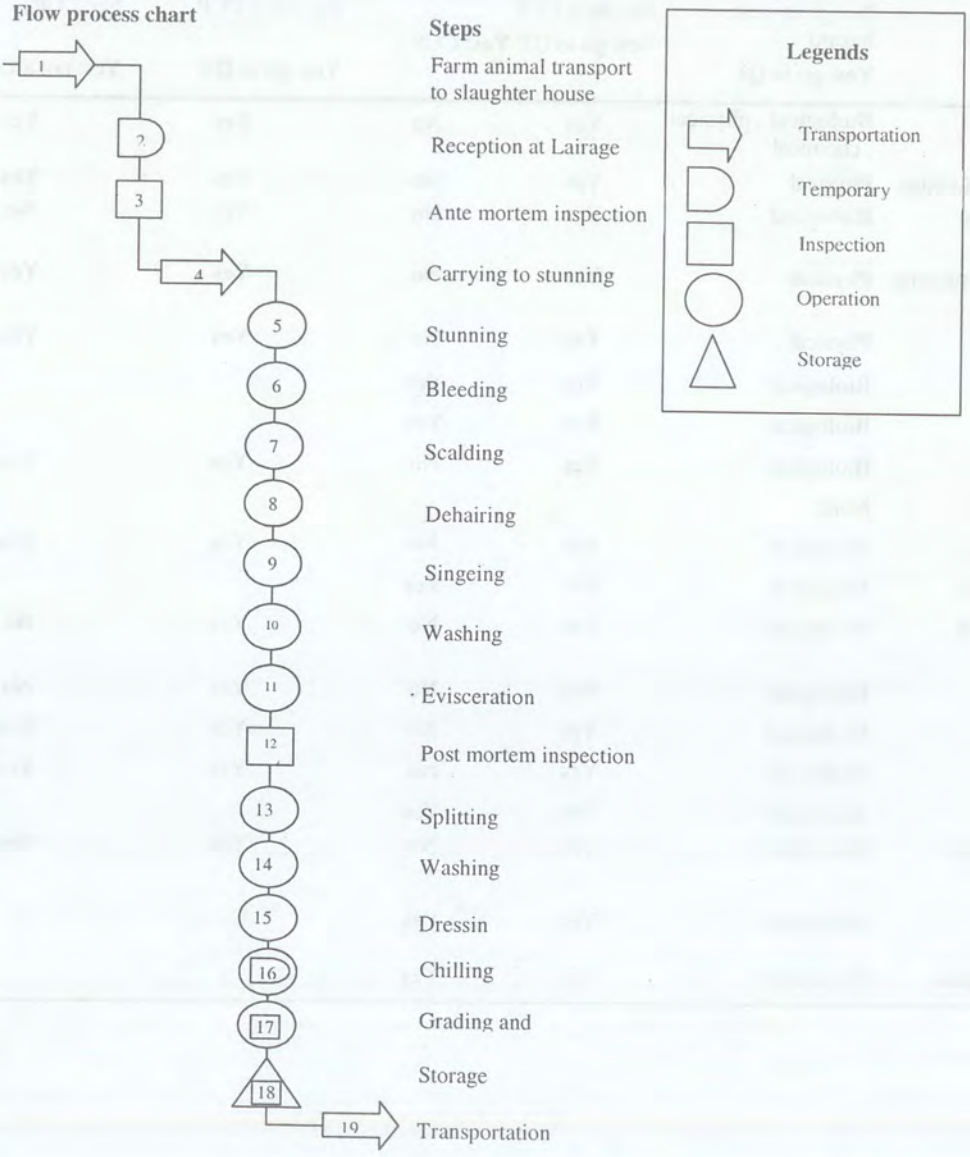

Fig 1: Proposed processing steps and flow process chart

Determination of proposed (CCPs)

The identification of CCP will be done according to the decision tree recommended by Codex (2004). The hazards and preventive measure which is necessary for determination of CCP is described in coming heading. CCP determination procedure for pig slaughtering is given on Table 2.

Table 2: CCP Determination on Pig Slaughtering

Processing steps	Identify hazard (physical, chemical, biological) Does hazard occur at this step / in this incoming material? No: go to next hazard Yes: go to Q1	Q1. Do preventive Measures exist for the identified hazard? No: not a CCP Yes: go to Q2	Q2. Does this step eliminate or reduce the likely occurrence of a hazard to an acceptable level? No: go to Q3 Yes: CCP	Q3 Could contamination with identified hazard(s) occur in excess of acceptable level(s) or could these increase to unacceptable levels(s) No: not a CCP Yes: go to Q4	Q4 Will a subsequent step eliminate Identified hazard(s) or reduce the likely occurrence to an acceptable level? No: CCP Yes: not a CCP	CCP number
1.Transport	Biological , physical , chemical	Yes	No	Yes	Yes	-
2.Reception at Lairage	Physical	Yes	No	Yes	Yes	-
3. Ante mortem inspection	Biological	Yes	No	Yes	No	1
4. Carrying to stunning pan	Physical	Yes	No	Yes	Yes	-
5. Stunning	Physical	Yes	No	Yes	Yes	-
6. Bleeding	Biological	Yes	Yes			2
7. Scalding	Biological	Yes	Yes			3
8. Dehairing	Biological	Yes	No	Yes	Yes	-
9. Singeing	None					-
10. Washing	Biological	Yes	No	Yes	Yes	-
11. Evisceration	Biological	Yes	Yes			4
12. Post mortem inspection	Biological	Yes	No	Yes	No	5
13. Splitting	Biological	Yes	No	Yes	No	6
14.Washing	Biological	Yes	No	Yes	Yes	
15. Dressing	Biological	Yes	No	Yes	Yes	
16. Chilling	Biological	Yes	Yes			7
17.Grading and packaging	Biological	Yes	No	Yes	Yes	-
18. Storage	Biological	Yes	Yes			8
19. Transportation	Biological	Yes	Yes			9

Details of HACCP plan: steps, identification of hazards, preventive measures (control measures), critical control points, critical levels (critical limits), surveillance (monitoring), corrective measure (corrective actions), and records

Step 1: Farm animal transport to slaughter house

Hazards: In this stage animals are driven from their point of origin to the slaughter house. Noise, loading and unloading, fighting due to the mixing of unfamiliar pigs and stocking too many pigs in transport vehicle causes severe stress for the animal resulting in impaired meat quality (Smulders & van Laack,1991). Surface contamination of animal by faeces is another hazard to be taken in to consideration. Microorganism mainly faecal in origin spread from carriers faeces to external surface of animal. Cross contamination between infected and healthy animals should not be underestimated, especially when they are damaged or bruised (FAO, 1991). The vaccines and antibiotics could remain in the edible tissue if animals after treatment are slaughter before the prescribed retention time for drugs (Alvarruiz-Bermejo *et al.*, 1999). Activation of the sympathetic nervous system in response to a stressor results in mobilization of energy (glucose) from storage (glycogen). Prolonged activation may leave little glucose available for conversion to lactic acid. The resulting meat will have a high pH (closer to physiological), a dark, dry appearance, reduced consumer appeal, a shorter shelf life, and (depending on duration) reduced levels of marbling. Activation of the sympathetic nervous system immediately prior to slaughter is particularly detrimental to swine. The fight response initiates glycogenolysis and elevates heart rate, blood pressure, and lactic acid in the muscle as the system converts to anaerobic metabolism. The accumulation of lactic acid results in a rapid pH decline while the carcass temperature is high. This leads to denaturation and shrinkage of myofibrillar proteins that bind water and development of PSE meat that has little consumer appeal and limited functional value in further processing (Bendall & Wismer-Pedersen, 1962; FAO, 1991; Subba, 2002; TLDP, 2003).

Preventive measures (control measures): Optimal stocking density will vary with transport time, genotype and climate and no standard values applicable for all types of journeys can be given. Transport trucks should be neither over nor under loaded. Overloading cause stress and bruising due to crushing. Under loading results in animal being thrown around and falling more than necessary. Driver should not corner at excessive speed and must accelerate and decelerate gently. Environmental temperature of approximately 16°C with a low air velocity results in best meat quality. Both during transport and lairage, mixing of unfamiliar pigs should be avoided since fighting and social stress in general lead to both more PSE and DFD (Karlsson & Lundström, 1992). In winter, care has to be taken that the animals do not become chilled as this is experienced as stress and leads to inferior meat quality. In order to reduce deaths during transport, feed is withdrawn before transport. Optimal feed withdrawal times are suggested to be in between 16 to 24 h or 12 to 18 h. If feed deprivation is too long (e.g. with overnight lairage), energy reserves are empty and there is not enough glycogen left to assure a sufficient pH decline and meat tends to become DFD (FAO, 1991; TLDP, 2003).

Critical levels: Animal Health and Livestock Services Act (1998) and Rules (2000) have made simple provisions for distribution (transportation) of live animals, however, no specific requirement stated on the Slaughter House and Meat Inspection Act (1999) and Rules (2001) on Nepal. In EU, America, Canada and developed countries, specific legislation are made on this regards. Standards are made regarding animal density, rest periods, environmental condition (protection from adverse weather, direct sunlight, adequate air and ventilation), feed and water and unloading parameters after arrival at lairage (Alvarruiz-Bermejo *et al.*, 1999). Similarly, proper sanitization of carrier vehicle should be done after unloading animal to prohibit the spread of disease and microorganism (FAO, 1991).

Surveillance (monitoring): Visual inspection of transportation condition, quarantine check post permission are the important monitoring aspects. Similarly, identification of shipments and control of documents needed for subsequent stages is very important. Each shipment should be accompanied by the appropriate documents in order to identify the animal, their origin, owner, departure and arrival places, date and time etc.

Corrective measures (corrective actions): These measures will be aimed at ensuring transportation by authorized vehicles and their compliance with the regulations and established norms.

Records: All the incidences occurring during transportation to the slaughterhouse will be recorded like followed route, stops, time during transport, feed given, rest given, temperature of environment and animal conditions at the time of receiving etc. These provide documentary evidence for future corrective measure.

Step 2: Reception and holding at lairage

Hazards: During unloading the stock from vehicle, stress can develop on the animal. Breaking of limbs and bruising of skin are common. These ultimately decrease the sensory and microbial quality of meat. Contaminated and diseased animal may spread disease to healthy animals and workmen.

Preventive measures (control measures): An unloading platform in inclined position made from concrete will minimize the physical hazard during unloading. The lairage should be shaded to minimize the environmental stress. Animals should be given proper amount of fed and water. A short lairage time of approximately 2 h at the slaughterhouse allows the animal to recover from transport stress and may improve both animal

welfare and meat quality. Showering the animals regularly during lairage, especially during the hot season, has a beneficial effect on aggressive behavior and welfare of the animals. Similarly, showering the animals before slaughter for ½ hour decreases muscle temperature and may lead to a better meat quality. Lairage should be separated from the slaughter hall and should have enough space to hold one day's stock. United Kingdom Ministry and Agriculture has specified 0.75 m^2 space per pig in lairage. Lairage should have separate inspection pen and suspect pen and any suspected animal should be immediately separated in to suspect pen after unloading. An electric goad rather than stick or tail twisting avoid stress on pig and also prevent carcass bruising. (Edwards *et al.*, 1979; FAO, 1991; TLDP, 2003)

Critical levels: All the animals admitted should be properly identified and documented and they should not have any clinical treatment recently. The animals that are too soiled or contaminated with faecal matter should be separated and washed thoroughly. Some requirements are expressed on Slaughter House and Meat Inspection Act and Rules like separate space for suspect and space for post mortem inspection of animal. The step is not regarded as effective CCP but is important and can be taken as partially effective CCP.

Surveillance (monitoring): Visual inspection will be carried out at the reception for assessing the condition and sanitary state of the animal. Condition of the reception area will be surveyed like type of floor, ventilation, light intensity, protections, water and feed supply, space allocated for each animal and separate area for unfamiliar animals. The disposal procedure of animal faeces, dead animal will be paid more attention.

Corrective measures (corrective actions): Whenever the animal arrives in inappropriate condition, the supplier will be warned and his authorization will be withdrawn if necessary. Other measures will be aimed at ensuring that the correct procedure and rest periods are implemented. Further more, special attention will be paid for cleaning and disinfection condition of the facilities.

Records: Animal reception documents and origin control will be recorded as well as the frequency and methods for cleaning and disinfection. The reception area and the corrective measures taken (retention of tired or excited animal, changes in water supply, etc.) will be documented.

Step 3: Ante mortem inspection

Hazards: The aim of ante mortem inspection is to segregate healthy animals from the unhealthy ones and prevent the slaughter of down, diseased, disabled and dead animals which are not fit for consumption. Also on the humanitarian ground, pregnant animals are refrained from slaughtering. Hazardous zoonotic disease like flukes, tuberculosis, anthrax, rabies, foot and mouth disease may be prevalent on the pig intended for slaughtering.

Preventive measures (control measures): The spread of zoonotic disease can be controlled on farm, if proper care is given to animal. Regular checking by veterinarian, regular vaccines and appropriate medicines check these diseases. The suspected animal should not be mixed with other healthy animal and man in contact with these un-healthy animals should also take proper precaution. Immediate separation of suspects should be done after unloading of animal so that the threat of diseases spread is minimized. The further actions taken to the suspect should be done in consolation with expert e.g. veterinarian or other as authorized by law.

Critical levels: The step is regarded as critical control point as if diseased animal are escaped from the inspection, it is sure that these diseased meat may be consumed by customer and probably spreading of zoonotic disease. Meat act/rule 1999, make it mandatory for each animal for antimortem inspection by approved meat inspector. None of the animal should have any type of zoonotic disease.

Surveillance (monitoring): Visual inspection will be carried out at the lairage. Generally a veterinarian examines the animal for disease by temperaturing and palpation and studying the movement and consistency of faecal discharge. Suspects may be detained and thoroughly examined. Depending upon the type and severity of case, an animal may be rejected and its carcass condemned. Ante mortem inspection is normally valid up to 24 h.

Corrective measures (corrective actions): Whenever the diseased animals are found, they are immediately separated. The government authority should be immediately informed through the proper channel, such that preventive measures can be taken at farm level. Awareness of the farmer regarding the disease plays more importance role in this stage.

Records: Animal health condition will be recorded. Treatment or other actions taken for suspects will be recorded. The cause of rejection, the symptoms of animal and condemned method will be properly recorded.

Step 4: Carrying to stunning section

Hazards: Beating and other stressful activities while driving to the slaughter hall causes stress and bruising in pig causing PSE condition and spread of microorganism.

Preventive measures (control measures): Lairage should be separated from slaughter hall. Pig race is designed in such that one animal is driven to slaughter hall at time. The race should be made slightly sloppy to facilitate forward movement of animal. It is recommended to provide foot bath in the race to wash the feet of animal (Subba, 2002).

Critical levels: This step is not regarded as the perfect or effective critical control point. The slopes of race and passage condition are to be as per standard. The animal should not

have any stress while moving to the slaughter pan. Control will be achieved in this and previous steps such that only healthy and clean animal are moved to the stunning pen.

Surveillance (monitoring): Visual inspection of movement of the animal to the stunning pan, and condition of foot bath water will be monitored.

Corrective measures (corrective actions): Whenever, any faulty activities on previous steps are suspected, the animals are detained from stunning, until finalization of suspected problem.

Records: Time taken for animal to reach the pan, change of water at foot bath etc will be recorded.

Step 5: Stunning

***Hazards*:** Pigs are stunned by electrical means. The aim of stunning is to make animal unconscious for bleeding. The hazard arising form inadequate stunning is stress on animal causing PSE meat and poor quality meat. Some time animal become violent if they are not properly stunned. If the consciousness broken in time of bleeding, the animal may carry sever physical harm. Collapsing of animal on floor may case the limb fracture. The result is bloody bone joints and bloody meat around bones and ribs. The increase in blood pressure and rupture in blood vessels resulting in short dark streaks called blood splash. Similarly, falling of larger size pig resulting in tearing (masceration) of muscle also result similar to blood splash.

Preventive measures (control measures): Proper training of stunner and adequate facilities for stunning plays a vital role. Provisions will be made in case of failure of animal to be stunned at first time or recover from consciousness in course of bleeding. Stunning can be done when pig is freely standing on the pan. In other case the animal is restrained in 'V' shape restrainer; the legs are strangled and squeezed. Proper maintenance equipment used for stunning is important. During electric stunning of pigs, electric contact is impeded by hair and caked mud. Water or brine will improve contact but the head must not be completely wet, otherwise the current will have a short circuit path avoiding the brain. The electrodes must be applied with strong pressure (FAO, 1991; Subba, 2002).

Critical levels: The stunning will be done by applying electric tongs on pig at ear to ear position or eye to ear position. The 50 Hz, 1.25 amp and 240 volt current is recommended for 10 second (Subba, 2002). Other various combinations can be used. Incase of cardiac arrest high voltage i.e., more than 250 volt is used and one tong is placed on the head and other on the body. Strict safety regulations should be maintained during stunning.

***Surveillance (monitoring)*:** Special attention will be paid to compliance with the norms in order to avoid incomplete stunning or undue suffering and stress on the pig. Visual inspection of stunning, condition of stunner, adequacy of ampere and voltage of electric current, position of tong, falling phenomenon of pig etc will be monitored. Similarly, preventive maintenance schedule, training of workmen etc will be monitored.

Corrective measures (corrective actions): Whenever the animals are not stunned on first application, immediate second application will be given. Immediate measures will be taken if the become violent due to improper stunning.

Records: Time of current application, preventive maintenance of stunner tong, workman training program, improper stunning etc will be recorded.

Step 6: Bleeding

Hazards: The objective of bleeding is to kill the animal with minimal damage to he carcass and to remove quickly as much blood as possible, as blood is an ideal medium for growth of bacteria (FAO, 1991). Bleeding is performed by cutting at least one of the carotid arteries as well as other surrounding vessels such as jugular vein (Alvarruiz-Bermejo *et al.*, 1999). In pig the sticking point is centre of neck just in front of breastbone. The main hazard will be contamination by the slaughter knife which could introduce microorganism form the animal skin. Another hazard taken into account will be poor bleeding and its later clotting. Yet another hazard is from the injury of trachea and oesophagus. The injury of trachea will lead to aspiration of blood in the lungs and the injury of oesophagus leads to contamination of blood and the sticking point. Too far sticking injures the shoulder with the formation of blood pockets. Too wide sticking wound leads to the contamination of pork carcass with the scalding water (Subba, 2002).

Preventive measures (control measures): The focus should be given to proper sterilization of sticking knife. Bleeding should be carried out with the animal in upright position to avoid the blood accumulation. The training of workmen plays vital role for appropriate bleeding operation of pig.

Critical levels: This step is regarded as effective critical control point. Any improper bleeding operation such as injury to trachea and oesophagus lead to unhygienic meat. Good manufacturing practices are required, paying special attention to replacement and sterilization of knife and general cleanliness of the operation.

***Surveillance (monitoring)*:** Cleaning and disinfecting condition of the facilities, equipment and tools will be monitored similarly, proper workmen procedure, and maintenance of general hygienic principle will be monitored.

Corrective measures (corrective actions): Focus will be given on the change frequency of knife and if necessary review of the hygiene and cleaning guidelines. If the oesophagus found to be injured, such carcass should be condemned or further

processed after through washing with chilled water after evisceration

Records: All the corrective measures will be recorded and documented. Further more, the training actions undertaken by the firm will also be recorded. The incidence of injury of trachea and oesophagus and condemned carcass will be recorded.

Step 7: Scalding

Hazards: During this stage, pigs are dipped in hot water (60-65 °C) for 5-6 minutes, to loosen the hair in follicle, which in principle reduces the surface contamination. In this stage the main hazards will be cross contamination of the surface by the reused scalding water and internal contamination by penetration of water from the sticking wound and relax breathing of animal (Troeger &Woltersdorf, 1990; Subba, 2002). Large numbers of bacteria generally of genus *Bacillus* capable of growth at high temperature had been found in almost all samples of water used in vat scalding of pig carcasses (Sorqvist, 1988).

Preventive measures (control measures): In pig slaughtering, the scalding process remains the unsolved problems as far as the meat hygiene is concerned (Troeger & Woltersdorf, 1990). Showering of carcass between bleeding and scalding not only reduces contamination of the carcass but also reduces the pollution of scalding tank water. During the process, the tank water becomes more and more contaminated. Therefore regularly emptying of the tank and refilling them with potable water is required. Similarly, pigs should be as clean as possible at sticking, and the bleeding should be fully completed before immersion (FAO, 1991). From bacteriological point of view, it is important that scalding temperature exceeds 60°C. Below 60°C the number of bacteria increases substantially. The scalding temperature should, on the other hand, not exceed 62°C, because of the coagulating effect on pig skin, resulting in subsequent damage to the skin by the dehairing machine. By scalding at the right temperature the number of aerobic CFU / cm^2 is reduced by 2 log cycli (Snijders, 1988). To avoid the penetration of water from the sticking point, plugging of the sticking point can be done (Subba, 2002). Instead of using batch method i.e., dipping in thermostatically controlled water tank, it is advisable to spray the hot water, which avoids the cross contamination compare to previous method. The pig can be transported through the tunnel and scalding can be carried out by the hot steam condensate (Troeger & Woltersdorf, 1990).

Critical levels: Water temperature should not be below 60°C to keep microbial count low. The frequency of change of water is important.

Surveillance (monitoring): The cleanliness of carcass before scalding, the temperature of tank, the frequency of change will be monitored. The equipment, tools and general hygienic measures followed by workmen will be monitored to ensure their cleanliness and correct operation.

Corrective measures (corrective actions): The under and over scalded carcass will be further processed after consultation with HACCP team. The maintenance of temperature will be corrected if found uncontrolled. Operating procedure and cleaning guidelines will be reviewed when control show that operations are not performed in optimal conditions.

Records: All the corrective measures taken, scalding water temperature, frequency of change of water, condition of carcass etc will be recorded.

Step 8: Dehairing

Hazards: In this stage most of the hairs are removed by knife and bell scrapper. Automatic machine carrying both scalding and dehairing operation is also available. The machine has the rotating arms with hard rubber tipped paddles (beaters). Post dehairing is done with knives to remove hairs from head, feet and skin folding, which are not removed during mechanical dehairing process. The main hazards will consist of possible cross contamination due to machine or scrapper or knife. The damage in carcass skin may induce internal contamination too.

Preventive measures (control measures): Preventive action will comprise good hygiene practices, as well as equipment and tools maintenance to achieve complete removal of hair which readily harbors microorganisms. Continuous training of workmen will prevent faulty operation.

Critical levels: These are defined by good manufacturing practices and satisfactory hygienic conditions as well as correct equipment operation.

Surveillance (monitoring): Visual control on operation and control of the cleanliness of equipment and workmen will be performed to ensure hygienic conditions.

Corrective measures (corrective actions): Operating procedure and cleaning guidelines will be reviewed when control showed that operations are not performed in optimal conditions.

Records: All the corrective measures applied and the reports of visual control of the operations will be recorded.

Step 9: Singeing

Hazards: Singeing may be used to remove any remaining hair and bristle after dehairing. The hairs are brunt with flame, where by the skin shrinks, close pores and sets leaving attractive clean appearance. Bacterial count on the skin is also reduced. Potable gas or kerosene torch will be used for singeing in small production. Singeing ovens are available for high throughput. The advantage of singeing lies in the decreased possibility of microbial growth due to heat and drying of skin. The possible hazard is cooking of skin, if over heat or long time is applied (Snijders, 1988; FAO, 1991; Subba, 2002)

Preventive measures (control measures): The preventive measures include proper maintenance of torch and training of workmen.

Critical levels: The carcass should be devoid of hair after singeing.

Surveillance (monitoring): Visual control on operation will be performed.

Corrective measures (corrective actions): Continuous training of workmen will prevent faulty operation. Cleaning guidelines will be reviewed when control show that operations are not performed in optimal conditions.

Records: All the corrective measures applied and the reports on the visual control of the operations will be recorded.

Step 10: Washing

Hazards: The final washing of the singed carcass will be done to remove the black brunt hairs and deposits. This will also reduce the microbial load of the carcass surface and prepare for safe evisceration.

Preventive measures (control measures): Preventive measure will comprise good hygiene practices, as well as equipment and tools maintenance to achieve complete removal of black deposit which obstructs evisceration. Continuous training of workmen will prevent faulty operation. Recognizing the limited contribution of cold water washing, a number of studies have reported that wash water temperatures of 85°C or higher are necessary if a decontamination effect is to be obtained. Experiments with washing carcasses for 20 s with water at 85°C have been reported to yield a 2 $\log_{10}$ cfu cm^{-2} reduction in *E. coli* and *Salmonella typhimurium* numbers (Gill *et al.,* 1995; Van Netten *et al.,* 1995). Commercial trials of a system to treat polished, un-eviscerated pig carcasses with sheets of water at 85°C for 15 s have achieved similar reductions in bacterial numbers, suggesting the clear value of such interventions as part of more effective HACCP-based control systems (Gill *et al.,* 1995). In more general terms, the decontamination effects of such washing could be enhanced by the inclusion of additional wash components. For example, studies have established that the addition of chlorine (up to 25 $\mu g\ l^{-1}$) or tri-sodium phosphate to the wash water can significantly decrease *Enterobacteriaceae* counts (Whyte *et al.,* 2001).

Critical levels: The water temperature used for washing should not be less than 60°C. The bacteriological quality of water is important.

Surveillance (monitoring): Visual control on operation and control of the cleanliness of equipment and workmen will be performed to ensure hygienic conditions.

Corrective measures (corrective actions): Continuous training of workmen will prevent faulty operation. Cleaning guidelines will be reviewed when control show that operations are not performed in optimal conditions.

Records: All the corrective measures applied and the reports on the visual control of the operations will be recorded.

Step 11: Evisceration

Hazards: In this stage viscera will be drawn from the abdominal cavity. The main hazard will be external and internal carcass contamination due to the high probability of contact between the intestinal content and the carcass if the alimentary canal is severed or if its contents overflow when it is being drawn from the abdominal cavity. Knife contamination will be common, and would lead to cross contamination between carcasses. Another problem to be coped with this stage is carcass soiling with intestine debris. All the viscera must be identified with the carcass until the veterinary inspection has been passed.

Preventive measures (control measures): The main preventive measure will be good hygienic practices associated to the operation (avoid cutting the intestine and viscera, removing of viscera and the hygienic of the tools and workmen) and proper training of workmen for adequate evisceration. Some measures are also specified on Animal Slaughter House and Meat Inspection Act/Rule. Similarly, FAO (1991), Subba (2002), and TDLP (2003) have prescribed methods for evisceration of pig. According to Snijders (1988), the good evisceration techniques for pigs are:

- Make small incision in the abdominal part of the belly. Put your fist which hold the knife upside down through the incision and open the carcass from the inside towards the outside, thus preventing damage to the intestine by pushing them away with the first
- Only cut the rind in the middle line and do not cut the omemtum , but break it by hand
- Cut out the rectum widely and subsequently tie it up, or
- Put the rectum into the ligamentae of the bladder in order to get a natural protection bag.

It is important to remove quickly both the giblets and inedible viscera after evisceration operation. Feed withdrawn from animals prior to slaughter will minimize the rumen content and possible hazards.

Critical levels: A sufficient slaughter cadence will be necessary, as well as the observance by the workmen of the above mentioned working condition: avoiding contact with intestine contents, proper and quick offal removal, etc. Satisfactory hygienic conditions will be needed. Temperature of water used for knife cleaning will be 80°C - 84°C. *Enterobacteriaceae* count and pathogens like *salmonella* count gives the indication of faecal contamination during evisceration (Snijders, 1988)

Surveillance (monitoring): Visual control on operation and control of the cleanliness of equipment and workmen will be performed to ensure hygienic conditions.

Corrective measures (corrective actions): The slaughter cadence will be corrected and the hygienic guidelines will be revised when necessary. In case of rectum and intestine content outflow, super-chlorinated water will be used for cleaning and soiled zones will be expurgated. Continuous training of workmen will prevent faulty processing as regards evisceration by causes of lack of ability.

Records: All the corrective measures applied and the incidences will be recorded, as well as the knife cleaning water temperature.

Step 12: Postmortem inspection

Hazards: Postmortem inspection consists in detecting viscera or part of them harboring infecting forms of zoonosis. The main hazards will be admission of carcass which should be deemed unacceptable.

Preventive measures (control measures): The main preventive measure will be unmistakable identification of viscera and carcass. The proper training of workmen regarding the common problems seen like tumors, cysts or parasites will increase the postmortem inspection efficiency. Factory workmen must not remove any diseased part until they have been observed by the inspectors; otherwise they may mask a general condition which should result in whole carcass condemned. Any instruction from the inspector to remove and destroy certain parts must be obeyed. Hazards naturally present like heavy metals of geogenic in origin, or induced by man like DDT, Lindan and HCB, chlorinated hydrocarbons, medicinal drugs, performance enhancer like copper added to pig feed can also be present on the pig meat in excess. Hecht (1988) has described the ways in which harmful residue get into the meat. Identification of these hazards need detailed chemical analysis of meat.

Critical levels: None of the carcass will be further processed unless it is inspected and approved by the inspector. Proper methods to separate suspected carcass and minimization of cross contamination are also important.

Surveillance (monitoring): Visual inspection will be performed in accordance to Animal Slaughter House and Meat Inspection Act (1999).

Corrective measures (corrective actions): The main corrective measures will consist in turning down the products which are deemed unacceptable. If necessary, operation will be redesigned adjusting them to the established hygienic and postmortem inspection guidelines.

Records: All corrective measures taken will be recorded, as well as rejected carcass, cause for rejection and lot identification.

Step 13: Splitting

Hazards: The main hazards arise from cross contamination by the used knife, saw and cleaver. Similarly, the contamination from workmen may take place.

Preventive measures (control measures): Comply with the personnel hygienic and splitting methods and sterilization temperatures of instruments will prevent the hazards.

Critical levels: The faulty process may damage the meat and makes unsightly. The water temperature should be maintained above 82°C for proper sanitization of cutting instruments.

Surveillance (monitoring): Visual control on operation and control of the cleanliness of equipment and workmen will be performed to ensure hygienic conditions.

Corrective measures (corrective actions): The slaughter cadence will be corrected and the hygienic guidelines will be revised when necessary.

Records: All the corrective measures applied and the incidences will be recorded, as well as the cutting instruments cleaning and sanitization water temperature

Step 14: Washing

Hazards: The primary object of the carcass washing is to remove visible soiling and blood stains and to improve the appearance after chilling. This washing step may spread the bacteria in the carcass rather than reduce the total number.

Preventive measures (control measures): Preventive measure will comprise good hygiene practices, as well as the bacteriological quality and temperature of water. Since, hot water can not be used in this step; washing with chilled water will minimize the surface microbial load and spread of microorganism. The uses of chlorinated water minimize the microbiological load on the carcass surface. Similarly, lactic acid treatment of carcasses, prior to chilling, has been shown to reduce bacterial levels by up to 3 log cycles (Gill *et al.*, 1995).The particular attention will be paid to the internal surface, the sticking wound and pelvic region. Bubbling of the subcutaneous fat may be caused by spraying with water at excessively high pressure, which may be due to the pressure in the system or a result of holding the spray nozzle too close to the carcass.

Critical levels: The microbiology and temperature of water, pressure applied is critical. Wiping cloth must not be used. The washed and dressed carcass must be immediately chilled to minimize the growth of microorganism.

Surveillance (monitoring): Visual control on operation and control of the temperature and pressure of water will be performed to ensure hygienic conditions.

Corrective measures (corrective actions): The slaughter cadence will be corrected and the hygienic guidelines will be revised when necessary.

Records: All the corrective measures applied and the incidences will be recorded, as well as the water temperature and pressure.

Step 15: Dressing

Hazards: The object of carcass dressing is to remove all damaged or contaminated parts as to standardize the presentation of carcasses prior to chilling, storage and final dispatch. Specification will differ in detail for different authorities. The hazard in this step will be contamination of carcass by the cutting knife or workmen.

Preventive measures (control measures): Preventive measure will comprise good hygiene practices, as well as the proper sterilization of cutting instruments.

Critical levels: The dressed carcass should be free from loose meat or fat parts which makes carcass unsightly as well as harbor microorganism.

Surveillance (monitoring): Visual control on operation and control of the temperature used for sanitizing of cutting instruments will be performed to ensure hygienic conditions.

Corrective measures (corrective actions): The inadequately dressed carcass will be reprocessed or condemned.

Records: All the corrective measures applied and the incidences will be recorded, as well as the water temperature used for sanitizing of cutting instruments will be recorded.

Step 16: Chilling

Hazards: Dressed carcass should go into the cooler as soon as possible and should be as dry as possible. The object of this stage is to hinder the growth of microorganism of public health significance, in order to be sure that the number of microorganism is not too high and that their toxin production is limited, to prevent the occurrence of food born disease from the consumption of pig meat. The main hazard will be the microbial growth caused by chilling that is too slow.

Preventive measures (control measures): The main preventive measure will consists of chilling the carcass surface to 7^0C, as quick as possible (minimum of 12-16 h from postmortem inspection) (FAO, 1991), making sure that cold chain will not be broken at any movement. Failure to bring down the internal temperature quickly will result in rapid multiplication of bacteria deep in the muscle, resulting in off-odors and bone taint, making meat inedible. The dressed carcass must be hanged on rails and precautions will be taken that carcass never touch the floor. The hygienic condition of floor, walls and rails and thermometer used for measuring deep meat is very important. The rate of cooling at the deepest point will vary according to many factors including the efficiency of the cooler, the load, carcass size and fatness. High air speeds are needed for rapid cooling but these will lead to increased weight losses due to evaporation unless the relative humidity (RH) is also high. However, if the air is near to saturation point (100 % RH) then condensation will occur on the carcass surface, favoring mould and bacteria growth. A compromise between the two problems seems to be an RH of about 90 % with an air speed of about 0.5 m/s is appropriate (FAO, 1991). Condensation will also occur if warm carcasses are put in a cooler partially filled with cold carcasses. The cooler should not be overloaded beyond the maximum load specified by the manufacturers and spaces should be left between carcasses for the cold air to circulate. Otherwise cooling will be inefficient and the carcass surface will remain wet, favoring rapid bacterial growth forming slime. Once filled, a cooler should be closed and the frequency of door opening should be as little as possible to avoid sudden rises in temperature. When emptied, it should be thoroughly washed before refilling. Personnel, handling carcasses during loading and unloading operations, should follow the rules regarding their personal hygiene; undue working with carcass on the chiller room should be avoided (FAO, 1991; Subba, 2002; TLDP, 2003).

Critical levels: The chiller load, time taken to reach the optimum deep muscle temperature, air velocity, and relative humidity of air will be the critical control parameters. The specific parameters will be acted in accordance with FAO (1991) and Subba (2002). The constructions of chilling facilities are very important for proper operation and hygiene maintenance on chiller and are detailed by Edwards *et al.* (1979) for different capacity requirements.

Surveillance (monitoring): Temperature (measured using recorder thermometers in the chamber and puncture thermometer in the carcasses), relative humidity (measured by hygrometers) and air velocity (measured by anemometers scattered in the chamber) will be controlled according to the chilling guidelines.

Corrective measures (corrective actions): With in corrective measures, the important are correction of chilling guidelines and cooling conditions (temperature, humidity, and air velocity) and revision of the equipment maintenance plan.

Records: Records will be kept for the physical parameters control, the deep muscle temperature and the preventive equipment maintenance.

Step 17: Grading and packaging

Hazards: After chilling and before storing the carcasses, they will be graded based on the meatiness of the pork and packaged. The main hazards in this stage will be the possibility of microbial contamination during packaging operation or due to increase in carcass temperature.

Preventive measures (control measures): There is no specific requirement stated on Animal Slaughter House and Meat Inspection Act/Rule. However in Europe pork meat is graded as E, U, R, O, and P; based on the lean meat percentage on the carcass. The instruments used and methods of grading is given by Subba (2002). GMP will be necessary, as well as appropriate hygienic conditions and equipments and tools maintenance will be maintained. Proper cleaning and hygienic conditions of packaging is important.

Critical levels: The critical parameters are the maintenance of cold chain (never below 7 °C); good personnel hygiene during packaging and grading operation and the microbiological and hygienic condition of packaging materials and machines.

Surveillance (monitoring): Visual inspection of equipments and tools will be performed to ensure correct manipulation and enforcement of cleaning and disinfecting program.

Corrective measures (corrective actions): Products and packages which are deemed unacceptable are rejected. The correction of working conditions and cleaning and disinfecting program will be made whenever necessary.

Records: Corrective measures, equipment maintenance and room temperature will be recorded.

Step 18: Storage

Hazards: The properly chilled and packed (or unpacked) carcass is stored on the chilled storage room. The main hazards will be microbial growth due to inadequate temperature, humidity or stowage conditions.

Preventive measures (control measures): Preventive measures taken to compliance with refrigerated storage guidelines, preventive maintenance of the equipment, the proper application of the cleaning and disinfecting program and a appropriate distribution of the meat inside the rooms.

Critical levels: The temperature of deep muscle should be below 7°C, oscillation of room temperature should be small and room relative humidity will be below 90 %. Water condensed in the outer surface of the evaporators should be prevented from contacting the carcasses. The proper operation of the cooling equipment will be maintained.

Surveillance (monitoring): Storage condition in the room as well as carcass temperature, stowage performed by the workmen and the cleaning and disinfecting program will be controlled.

Corrective measures (corrective actions): Corrective measures will include; rejection of unacceptable products, revising the guidelines for hygienic storage and set up for preventive plan for equipment maintenance.

Records: All the corrective measure taken, room temperature, relative humidity, preventive maintenance of equipment, input and dispatch of carcass etc will be recorded.

Step19: Transportation

Hazards: The main hazards will be possible contamination during transportation and increase in microbial load due to fluctuations in temperature during transportation.

Preventive measures (control measures): Vehicles for transporting meat and carcasses should be considered as an extension of the refrigerated storage. The objective must be to maintain the meat temperature at or near 0°C. Meat should be chilled to 0°C before loading. Meat should hang on rails, not on the floor. If stockinettes are put on carcasses they must be clean. Meat trucks should not carry anything other than meat. Insulated vans without refrigeration may be refrigerated by adding dry ice. While this is a reasonably good alternative to the refrigerated truck, it does not allow the temperature to be controlled. Un-insulated vans and open trucks should not be considered as suitable transport for meat, particularly in hot climates. In addition to the temperature abuse, condensation will occur when the meat goes back into refrigeration and in open trucks the meat is exposed to attack from insects. Loading and unloading should be done quickly. If there are any unavoidable delays then dry-ice blocks should be placed in the partly filled van.

Critical levels: The temperature of vehicle should be near 0°C through out the route. Vehicle hygiene and stowage will also be of paramount importance to avoid accessory contamination.

Surveillance (monitoring): Storage condition (temperature, cleanliness and other hygienic measure) in the vehicle will be monitored.

Corrective measures (corrective actions): Corrective measures will include: correction of hygienic and stowage conditions, as well as the cleaning and disinfecting program.

Records: All the corrective measure taken, vehicle temperature, input and dispatch procedure of carcass etc will be recorded.

Application synoptic of HACCP plan in pig slaughtering process

The HACCP plan during pig slaughtering is summarized on Table 3

Concluding Remarks

HACCP is an effective precautionary control system that, if applied correctly and systematically, offers the means of identification and assessment of any possible physical, chemical and microbiological hazards, the detection and control of critical points in all food production steps. It is a recommended food safety system by Codex and is adopted by ISO 2200: 2005 on its Food Safety Management systems-requirement for any organization on the food chain (ISO, 2005). In these days, as the food safety is becoming very sensitive issues and HACCP is getting tremendous attention among food producer and traders. Compliance with HACCP system can be volunteer activity of organization to improve its food safety system or it can go on third party certification. The HACCP certification will create more assurance to consumer, so can be a marketing tool. The adoption of the system may need investment initially to maintain the plant and procedures that comply with the requirement and training of the workmen, however in long run, increased consumer satisfaction and lesser consumer complaints form safe food will overcome the initial investment and finally HACCP wouldn't cost rather pays.

Table 3: HACCP Plan on Pig Slaughtering

Hazards	*Preventive (control) measures*	*CCP*	*Critical levels*	*Surveillance (monitoring)*	*Corrective measures (actions)*	*Records*	*Verification s*	*Selected references*
Stress on pig (DFD, PSE), surface bruising , cross contamination, and microbial migration	Vehicle cleaning and disinfecting, gentle acceleration deceleration of vehicle	No	Legal norms for animal welfare	Transportation conditions, batch identifications, documentation control	Ensure transportation in authorized vehicles, compliance with norms	Incidence during transportation, followed route	HACCP team member	FAO (1991), Alvarruiz-Bermejo *et al.*,(1999)
Stress, injuries, soiling of animal and cross contamination	Design of unloading platform , shading of lairage, showering of animal, proper feed and water	No	Turndown of soiled and diseased animals, identification of lot	Visual inspection of animal, disposal procedure of animal faeces, and dead animal	Warning of supplier when inadequate condition is seen, proper rest to stressed animal	Reception documentation , animal origin, corrective measures	HACCP team member	Subba (2002).
Biologically diseased pigs	Trained and qualified inspector employed and approved by regulatory authority, awareness and training of farmers at farm level, inspection normally valid up to 24 h	Yes	Non of the diseased animal enter the processing line	Visual inspection of movement and consistency of faecal discharge, temperaturing, and palpation	Immediate separation of suspects	Animal health condition, treatment and other actions, cause of rejection	Meat inspector and HACCP team leader	Meat Act (1998), and Rule (2000)
Stress and bruising in pig	Lairage design, design of pig race, providence of foot bath	No	Slope of race , no stress to animal	Movement of animal, condition of foot bath	Suspects are detained from stunning	Time taken by animal, change of foot bath water	HACCP team member	Chamber & Grandin (2001)
Stress or injuries, incomplete stunning	Proper equipment operation and maintenance, hygiene of animal	No	Voltage, ampere, frequency of current, position of tong, pressure and time of application as per guidelines	Visual inspection, compliance with established norms	Restunning	Operational records, preventive maintenance schedule	HACCP team member	Subba (2002), FAO (1991)
Deep meat contamination by tools, poor bleeding	Frequent change bleeding knife , bleeding in upright position	Yes	No injury to trachea and oesophagus, sanitization of bleeding tools as per guidelines	Cleaning and disinfecting condition of tools, equipment and facilities, adoption of proper procedure	Knife/tools change frequency, revision of cleaning and disinfecting guidelines	Corrective measures, training actions	HACCP team leader	FAO (1991), and Subba (2002)
Microbial contamination from scalding water	Scalding methods, water renewal, temperature control	Yes	Water temperature between 60-62 °C, microbial condition of water as per established norms	Temperature, renewal and steady flux of water, checking of equipments and tools	Change of working procedure, revise cleaning and renewal schedule	Corrective measure, scalding water temperature and time	HACCP team leader	FAO (1991), Subba (2002)
Microbial contamination, damage to carcass skin	Proper adherence to the guidelines and good hygienic practices	No	Good manufacturing and hygienic practices	Visual control, cleaning condition of workmen and equipment	Correction of existing procedure, revise the operation guidelines	Corrective measures, time taken per animal	HACCP team member	FAO (1991), Subba (2002)
Cooking of skin	Proper adherence to the guidelines and good hygienic practices	No	Time per animal, others as defined by guidelines	Visual control	Revise of guidelines	Corrective measures, time taken per animal	HACCP team member	FAO (1991)
Spread of microorganism	Proper adherence to the guidelines and good hygienic practices	No	Microbial quality and temperature of spray water as per established standards	Visual control	Revise of guidelines	Corrective measures, time taken per animal	HACCP team member	FAO (1991)
Microbial contamination	Proper training of workmen for adequate evisceration, good hygienic practices	Yes	Standards for microorganisms	Control of operation, cleaning and disinfecting condition, offal removal and disposal	Correction of working condition, carcass cleaning in case of digestive content out flow	Corrective measures, time taken per animal, sanitization of tools and operating procedure	HACCP team leader	FAO (1991), Snijders (1988)

Admission of unacceptable carcass	Adequate training of workmen regarding the possible defects, identification of offals and carcass, avoidance of cross contamination	Yes	Standards given in acts and rules	Visual inspection by veterinarians or meat inspector	Carcasses turndown and redesign of operations	Corrective measures, seized or turndown carcass	HAC CP team leader/ meat inspect or	Meat Act (1998) and Rule 2000
Cross contamination by tools, physical hazards by bone particles	Comply with the personnel hygienic and splitting methods and sterilization temperatures of tools	Yes	Defined by good manufacturing and hygienic practices	Visual control on operation and control of the cleanliness of equipment and workmen	Correction of slaughter cadence, revision of hygienic guidelines	corrective measures applied, tools cleaning and sanitization water temperature	HAC CP team leader	FAO (1991), TLDP (2003)
Spreading of bacteria	Use of chilled water, using appropriate pressure	No	Microbiology and temperature of water, pressure applied should be per standard	Visual control on operation, control of temperature and pressure of water	Correction of slaughter cadence, and revision of hygienic guidelines	Water temperature and pressure, corrective measures	HAC CP team memb er	FAO (1991), Subba (2002)
Contamination of carcass by the cutting knife or workmen.	Good hygiene practices, as well as the proper training of workmen	No	Defined by good manufacturing and hygienic practices	Visual control on operation	Correction of slaughter cadence, and revision of hygienic guidelines	Corrective measures	HAC CP team memb er	FAO (1991), TLDP (2003)
Microbial growth caused by slow chilling	Good hygiene practices, as well as the proper preventive maintenance of chiller system, and training of workmen; fast chilling as far as possible	Yes	Chilling to 7 °C, with in 12-16 h from postmortem inspection, other as per good manufacturing and hygienic practices	Air velocity, relative humidity, temperature and chilling guidelines	Correction of chilling guidelines and cooling conditions; revision of the equipment maintenance plan	Physical parameters control, deep muscle temperature, preventive equipment maintenance.	HAC CP team leader	FAO (1991), Subba (2002), TLDP(20 03)
Microbial contamination , improper grading	Good hygiene practices, proper selection of packaging material, preventive maintenance of equipment	No	Grading according to meatiness , adherence to standard operating procedures , room temperature below 12°C	Visual inspection of equipments and tools, room temperature control	Rejection of deemed unacceptable product and packages, correction of hygienic programs	Corrective measures, equipment maintenance, room temperature	HAC CP team memb er	Subba (2002), , TLDP (2003)
Microbial growth and cross contamination	Compliance with refrigerated storage guidelines, proper application of cleaning and disinfecting programs, appropriate stowage	Yes	Temperature of deep muscle below 7°C, RH below 90 %, proper cooling equipment operation, others as specified on the general hygienic guidelines	Visual inspection of room, room temperature control	Unsuitable products turndown, correction of storage and hygienic conditions, correction of equipment maintenance plan	Corrective measures, storage conditions, report on preventive equipment maintenance	HAC CP team leader	Subba (2002), , TLDP (2003)
Microbial contamination, inadequate transportation	Temperature control, hygienic measures on the vehicle and container	Yes	Transport temperature, load incompatibility	Visual inspection of vehicle and transportation methods practiced	Correction of hygienic stowage and transport condition, correction of cleaning and disinfecting programs	Corrective measures, refrigerated vehicle temperature	HAC CP team leader	Subba (2002), , TLDP (2003)

Few organizations are certified by HACCP in Nepal. However, majority food producers are still not able to maintain basic hygienic requirement for food safety like wearing of gloves on handling food products, wearing of aprons and boots on production area, sanitization of hands and equipment. Most of industries are under strict unhygienic condition. This is basically lack of awareness to the manufacturer and workmen rather than slackness. A small motivation and inspection by government regarding the compliance of hygienic measures as stated on Food Act/Rule and other associated legislations like consumer act will definitely improve the hygienic condition of our plants. Regarding meat, the act and rule enacted form 1999 and 2001, is seen functionless and meat trade is professed very unhygienic. Food habit of long cooking of meat before consumption is playing as protective aids from lethal microorganisms that are very prone to exist on meat slaughter

and marketed on unhygienic condition. However, hazards inform of toxins are still present on the cooked meat. Due to lack of data, it is still undetermined the effect of consumption of unhygienic meat on the public health. So, to safeguard the consumer health, the government, the meat producer and traders should take immediate action to systematize the haphazard and unhygienic current practice of slaughtering, transportation and retailing of meat and meat animal. At, least government should make it mandatory to carry slaughtering operation at specified locations and premises as stated on Slaughter House and Meat Inspection Act (1999) and Rules (2001). Considering fundamental right to get diseased free food (meat), at least antimortem and postmortem inspection of animal and carcass need to be carried out. If we start from these basic hygienic and safety principles, we can move forward to the compliance with HACCP guidelines. Nepal has maximum prospects for organic pig farming, and as a member of WTO, international meat trade is also open to us. Compliance with HACCP and third party certification will help for international export of our meat and meat products. Today, it is quite impossible to export food products, especially meat, to EU, America, Canada and other developed countries with out compliance with HACCP. It is emphasized on possible mandated imposition of HACCP in Pig Slaughtering on which differs from its use as a private process control method or as a private means of certification. Thus, it should be started by basic hygiene and sanitation principles and finally reviewing the concept of HACCP for its application on Pig slaughtering practices.

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Safety Aspects of Biogenic Amines (BAs) in Muscle Foods

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Biogenic amines are one of the major antinutritional factors abundantly found in varieties of foods and beverages including muscle food products. Histamine, tyramine, putrescine, cadaverine, tryptamine, â-phenylethylamine, spermine and spermidine are some common amines found in muscle foods. However, their levels of concentration in muscle foods may greatly depend on several physico-chemicals as well as biological factors. Since the amines are toxic compounds, their presence in muscle foods are considered to be a critical from a hygienic point of view as they have been implicated as the main causative agent in a number of food poisoning epidemics in human health including histamine poisoning as well as genetic predisposition, gastrointestinal diseases, inhibiting amino oxidases activity and so on . Apparently, contamination with amino acid decarboxylase bacteria either in raw material or processing or storage stages are critical control points (CCPs) to control the formation of different biogenic amines in muscle food products. Nevertheless, it is to be hoped that use of hygienic quality raw material, amino acid decarboxylase negative and/or amine oxidase positive meat starters, proper technological processing conditions and a mandatory food quality control system could produce hygienic quality of muscle food products that would have none or less health risk factors.

Keywords: Biogenic amines (BAs), Muscle foods, Amino acid decarboxylation, Amine oxidase activity (AOA), Safety

Introduction

Biogenic amines (BAs) are low molecular weight and basic nitrogenous organic compounds are formed mainly by decarboxylation of amino acids by microorganisms or by amination and transamination of aldehydes and ketones, which could be aliphatic, aromatic or heterocyclic in structure (Silla Santos, 1996). However, simple aliphatic monoamines are widespread. Diamines and polyamines can be found in animals, plants and bacteria (Smith, 1980). Since, amino acid decarboxylation in most of foods is caused by the action of living organisms, the metabolic products thus formed are termed as "biogenic amines" (Shalaby, 1996).

Amongst different amines histamines, putrescine, cadaverine, tyramine, tryptamine, â-phenylethylamine, spermine, spermidine and arginine are abundantly found in meat products (Vandekerckhove, 1977; Hernández-Jover *et al.* 1996; Durlu-Özkaya, 2001; Coïsson *et al.*, 2004). Besides meat products, other kind of foods and beverages e.g. fish products, eggs, milk, cheeses, chocolate, fermented vegetables, soy bean products, beer, wine and so on have been reported as to be contaminated with different kinds of BAs including serotonine, agmatine and dopamine (Smith, 1980; Shalaby, 1996; Silla Santos, 1996). Meat products except dry fermented sausages that have often been reported to be contaminated with BAs (Suzzi & Gardini, 2003), other meat products such as dry cured ham, cooked ham as well as hamburgers and ground meats also been reported to be contaminated with different amines (Shalaby, 1993; Hernández-Jover *et al.*, 1997; Durlu-Özkaya *et al.*, 2001). In Egyptian dry sausages putrescine and cadaverine were found in more than 90% of the tested samples while phenylethylamine only in 18 % (i.e. lowest) (Shalaby, 1993). Some important BAs that have been reported in different meat products are given in Table 1. This article is a critical review on safety aspects of biogenic amines in muscle foods.

Table 1 : Biogenic amines detected in some muscle food products in European markets

Biogenic amines	*"pur porc"[A] (mg/100g dry matter)[a]*	*Egyptian dry sausages (mg/kg)[b]*	*"chorizo"[B] (mg/kg)[c]*	*Italian sausages (mg/kg)[d]*	*Norwegian sausages (mg/kg)[e]*	*Hamburger (mg/100g)[f]*
Histamine	2.65 to28.59	7.5 to40.5	n.d. to 108.3	0	1	n.d.
Tyramine	31.92 to78.71	9.5 to52.8	76.5 to477.8	187	12	n.d. to 1.35
Putrescine	7.49 to 29.64	12.0 to102.4	31.6 to 361.9	1	1	n.d. to 1.23
Cadaverine	6.12 to 29.64	5.6 to 38.5	3.9 to 34.9	1	1	n.d. to 0.21
β-Phenyl-ethylamine	tr to 1.31	1.5 to 80.75	n.d. to 7.7	39	6	n.d.
Tryptamine	N.D.	2.5 to 33.25	5.7 to 65.1	19	9	n.d. to 2.7
Spermidine	N.D.	5.3 to 11.75	1.4 to 7.9	6	4	n.d. to 0.51
Spermine		1.5 to 5.25	15.4 to 37.8	18	24	n.d. to 0.34

[A]French air dried sausage; [B] Spanish dry cured sausage; N.D., No data; n.d., not detected; tr. trace amount
References: [a] Vandekerckhove (1997); [b] Shalaby (1993); [c] Hernández-Jover *et al.*, (1996); [d] Ansorena *et al.*, (2002); [e] Ansorena *et al.*, (2002); [f] Durlu-Özkaya *et al.*, (2001).

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Factors Influencing on the Formation of Biogenic Amines in Muscle Foods

Physico-Chemical Factors

Quality of raw meat

Normally, BAs are not present itself in meat exceptionally spermine and spermidine were detected initially in chicken meat (Silva & Gloria, 2002) and raw pork, which remained constant during processing and storage (Hernañdez-Jover *et al.*, 1996).The presence of BAs in meat products at high concentration could be associated with the use of poor hygienic quality meat and/or improper manufacturing practices. (Hernández-Jover, 1997; Silva & Gloria, 2002). Therefore, many authors have been suggested that a hygienic quality of raw meat is most important factor to control BAs in final products (Bover-Cid *et al.*, 2000; Coïsson *et al.*, 2004). Moreover, it has also been reported that white meat (chicken) are more susceptible than red meat (bovine) to form BAs due to shorter muscle fibres in white meat as compare to bovine muscles that may facilitate attack to prteolytic enzymes (Vinci & Antonelli, 2002).

Physico- chemical parameters

The pH drop during fermentation was significantly correlated with the formation of BAs in meat products. Lower the pH value in products exhibited lower level of amine content and *vice- versa.* At higher pH, most of spoilage bacteria can grow very well that could produce higher amines in meat products (Hernañdez-Jover *et al.*, 1996).Beside these, bigger size and inner portion of sausage possessed higher amount of tyramine (Bover-Cid *et al.*, 1999b). Komprda *et al.* (2004) have also been reported the similar finding regarding BAs in dry fermented sausages having bigger diameter.

Food additive and ingredients

There are several food additives such as sugar (Bover-Cid *et al.*, 2001a; González-Fernández, 2003), sodium chloride (Gardini, 2001), and spices particularly clove, cinnamon, sage and paprika etc (Wendakoon & Sakaguchi, 1992; Komprda *et al.*, 2004) have been reported as inhibitors to biogenic amines in different meat systems. As the antimicrobial action of sodium sulphite, the use of 500 to 1000mg/kg in sausage ripening could markedly inhibit the formation of different amines i.e. cadaverine, histamine, phenyl-ethylamine, tryptamine and agmatine. In contrast, tyramine and putresciene production were stimulated by the presence of sodium sulphite (Bover-Cid *et al.*, 2001b).

Processing and storage condition

High amount of tyramine was reported in dry sausages ripened at higher temperature as compare to low temperature ripened one (Maijala *et al.*, 1995a). Hernandez –Jover *et al.* (1997) reported that the lower level of BAs (10mg/kg) in cooked meat products, whereas ripened one reached over 300mg/kg.Thawing of frozen raw meat is an another meat processing step, which also could influence on the formation of BAs in dry sausages, however it depends upon the use of starter cultures (Maijala *et al.*, 1995b). Similarly, higher temperature favored in accumulation of BAs during storage of dry fermented sausages as compare to refrigerated one (Bover-Cid *et al.*, 2001c). Komprda *et al.* (2004) also reported the similar finding that higher levels of Bas were detected in sausages stored at room temperature. The effects of control, vacuum or modified atmosphere packaging on the formation of BAs have been elicited by different authors (Kaniou *et al.*, 2001; Ruiz-Capillas &Moral, 2002). The control and modified atmosphere packaging significantly inhibited formation of BAs except agmatine in *hake* samples (Ruiz-Capillas & Moral, 2002). Nevertheless, vacuum packaging could not exhibit such effect on beef stored at 4^0C. Since amines are formed on the surface of meat products, simply washing with clean water while they are removing from the package could reduce their level significantly (Kaniou *et al.*, 2001).

Biological Factors

Bacterial amino acid decarboxylase activity

In muscle food products different strains of *Pediococcus, Enterobacteriaceae, Lactobacillus, Streptococcus, Micrococcus*, and *Carnibacterum* species could produce biogenic amines during spoilage or fermentation due to their free amino acid decarboxylase activity (Table 2).

Higher amount of cadeverine, putrescine, and tyramine production were correlated either with high number of *Enterobacteriacae* or aerobic total counts (Hernañdez-Jover, *et al.*, 1996; Bover-Cid & Holzapfel, 1999). Bover-Cid *et al.* (2003) also found the similar relationship between the formation of cadeverine and *Enterobacteriaceae* in pork sausage. Likewise, higher level of BAs was formed in spontaneously fermented meat system (Bover-Cid *et al.*, 1999b; Bover-Cid *et al.*, 2000; Bover-Cid *et al.*, 2003). Due to higher proteolytic activity, the starter cultures produced more amine precursor free amino acids, which eventually facilitated to produce BAs during ripening of dry sausage (Bover-Cid *et al.*, 1999a). In other hand, mixed culture of *lactobacilli* and *staphylococci* inhibited the formation of putrescine in Turkish soudjoucks, but didn't to tyramine (Ayhan *et al.*, 1999). Maijala *et al.* (1995a) also reported significant reduction in the levels of histamine, tyramine and cadaverine during ripening of dry sausage with the mixed culture of *P.pentosaceus* and *S. carnosus*.This author has also found approximately double the quantity of histamine and 1.2 times more tyramine than the sample inoculated with *P.pentosaceous*- ATCC 33316 in Chinese style dry fermented sausage. Likewise, 2 times more histamine and 1.5 times more tyramine has been found in control sample (no added culture) as compare to the Chinese style dry fermented sausage inoculaed with with *L. casei* subspp. *casei* 1.001 (Rai *et al.*, 2005). This result was probably due to an antibacterial property of lactic acid bacteria (LAB) as the strains used in all samples

Table 2 : Different strains of biogenic amines producing bacteria isolated from meat and meat products

Source	*Starter*	*Main biogenic amines*	*Reference*
Sausages	*Kocuria varians, S. carnosus* ,	Phenyethylamine and tryptamine	Hernández-Jover *et al.*, (1997)
Fermented Pork sausages	Lactic acid bacteria (viz. *L. curvatus, L. brevis, L. bavaricus, L. sake, L. faecalis, L. paracasei etc.)* Enterobacteriaceae:(viz. *Klebsiella oxytoca, Serratia sp. Proteus vulgaris, C. freundii*)	Tyramine, putrescine, phenylethylamine, cadeverine and putre-scine	Bover-Cid *et al.*, (2001d)
Ground beef and Hamburgers	*E. coli*-EC04, *E. coli, E. aerogenes* and *K. pneumonia, Citrobacter freundii* and *Enterobacter* spp.	Histamine, cadaverine and putrescine	Durlu-Özkaya *et al.*, (2001)
Fermented Sausages	*Staphylococcus xylosus*	Spermine and/or sper-midine	Martuscelli *et al.*, (2000)
Fermented sausages	*Lactobacillus sake*-54, *L. pentosus*-R1, *P. pentosaceus*-19, *Micrococcus parvus*- M7	Tyramine, putrescine, cadaverine, histamine, spermine , spermidine	Zhang (2003)
Meat products	*Carnobactrium divergens, C. piscicola, L. farciminis, L. plantarum, L. curvatus*	Tyramine	Masson *et al.*, (1996)

showed lower total plate counts and *Enterobacteriaceae* counts (Rai, 2005). Since amino acid decarboxylase activity have been found strongly strain dependent rather than being related to specific species, the amine negative strain seems to a novel meat starter for processing of fermented muscle foods (Martuscelli *et al.*, 2000; Zhang, 2003).

Bacterial amine oxidase activity

Biogenic amines are physiologically inactivated by amine oxidases, which are enzymes found in bacteria, fungi plant and animal cells, able to catalyze the oxidative deamination of amines with production of aldehydes, hydrogen peroxide and ammonia. Fig 1 shows the sequential actions of an amine oxidase and aldehyde dehydrogenase to convert 2-phenylethylamine and tyramine into phenylacetic and 4-hydroxyphenylacetic acid respectively (Cooper, 1997). Different amine oxidase activity such as tyramine oxidase activity of *Micrococcus varians in vivo* (Leuschner & Hammes, 1998) and histamine oxidase activity of *Staphylococcus xylosus* strains *in vitro* and in meat system (Gardini *et al.*, 2002; Zhang, 2003) and tyramine and 2-phynylethylamine oxidase activity of *Klebsiella aerogenes* W70 *in* vitro (Cooper, 1997). In similar study, this author has also found histamine and tryptamine oxidase activity of *S. xylosus*-12 as the strain used sample has been found lowest level of amine as compare to control and LAB used bacteria (Rai, 2005). Hence, oxidizing ability of bacterial starter particularly histamine, tyramine and/or tryptamine seems to be another good criteria to select a hygienic strain for fermented meat products that could suppress the level of BAs in final muscle food products.

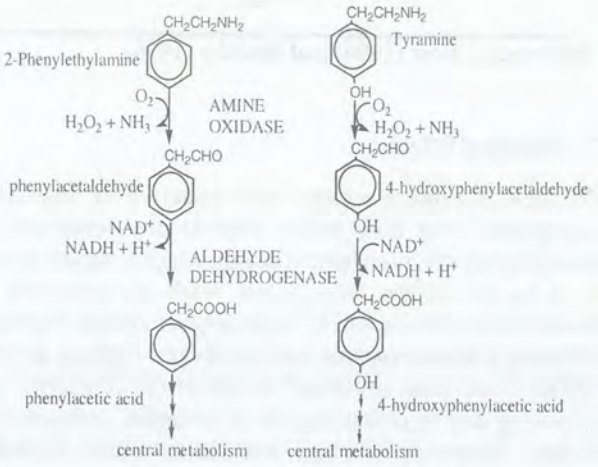


Fig 1 : Sehematyic representatios of the reaction involved in 2-phenyl ethylamine and tyramine catabolism

Reference: Cooper (1997).

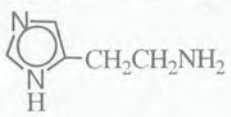
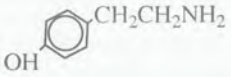
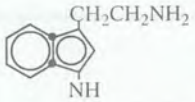
Analytical Technique

To analyze BAs in foods and beverages a number of analytical techniques have been reviewed by several authors such as liquid chromatography (LC), high performance liquid chromatography (HPLC), gas chromatography and GC-mass spectrometry, thin layer chromatography (TLC) and enzyme-linked immunosorbent assay system (ELISA) and so on (Silla Santos, 1996; Shalaby, 1996). More recently, capillary electrophoresis with laser induced fluorescence (CE-LIF)

technique with the detection limit up to 10^{-10} mol/l has been reported as a latest analytical technique for analyzing BAs more efficiently in plant and biological samples (Liu *et al.*, 2003).

However HPLC is most widely used technique to analyze BAs in muscle foods, TLC also seems to be more feasible technique because of its low cost as well as simplicity, rapidity and validity (Shalaby, 1999).

Table 3 : Major biogenic amines their structural formula and some important pharmacological effects

Biogenic amine	*Structural formula*	*Precursor*	*Pharmacological effects*
Histamine		Histidine	Liberates adrenaline and noradrenaline; Excites the smooth muscles of the uterus, the intestine, and the respiratory tract; Stimulates both sensory and motor neurons; Controls gastric acid secretion.
Tyramine		Tyrosine	Peripheral vasoconstriction; Increases the cardiac output; Causes lacrimation and salvation; Increases respiration; Increases blood sugar level; Release noradrenaline from the sympathetic nervous system; Causes migraine.
Putrescine and cadav-erine	$H_2N(CH_2)_4NH_2$ $H_2N(CH2)_5NH_2$	Ornithine and lysine	Hypertension; Bradycardia; Lockjaw; Paresis of the extremities; Potentiate the toxicity of other amines.
β-Phenyl-ethylamine		Phenylalanine	Releases noradrenaline from the sympathetic nervous system; Increases the blood pressure; Causes migraine.
Tryptamine		Tryptophane	Increases the blood pressure.

References: Nout (1994) and Shalaby (1996)

Toxicological Effect

Biogenic amines are either psychoactive or vasoactive compounds that may cause several physiological and toxicological affects on human health, which is shown in Table 3. BAs are often associated with an outbreak of scombrotoxicosis caused by consumption of fish especially of family *Scomberesocidae* and *Scombridae*. Lehane & Olley (2000) have been reviewed in detail the histamine fish poisoning due to comsumption of histamine contaminated fishes. However, dietary histamine alone failed to establishsignificant correlation with scombrotoxicosis in volunteers (Clifford *et al.*, 1991); though cadaverine could act as a potentiator to histamine in scombroid poisoning (Bjeldanes *et al.*, 1978).

From an animal (rats) study, the acute toxicity of tyramine, cadaverine and putrescine have been reported i.e. 2000mg/kg body wt. for spermidine, while 600mg/kg body wt. for spermine. Furthermore, no observed adverse effect level (NOAEL) has been reported as 180mg/kg body wt. for tyramine, cadaverine and putrescine, 83mg/kg body wt. for spermidine and 19 mg/kg body wt. for spermine (Til *et al.*, 1997).

The threshold toxic dose of individual amine has not been precisely defined but over1000ppm in muscle food is supposed to elicit its toxic effects on human health (Nout, 1994; Lehane & Olley, 2000). In addition, some primary and secondary amines such as putrescine, cadaverine, ornithine and lysine could react with residual nitrite in cured meat products forming several carcinogenic nitrosamines (Warthesen *et al.*, 1975; Smith, 1980).

Safety Level

Since, histamine is most toxic amine, its maximum permissible limit for fish and fish products have been set at the level of 200mg/kg of food in Germany but only 100mg/kg in Canada, Finland and Switzerland (Lange & Wittmann, 2002).

The precise hygienic standard of BAs in meat and meat products is not clear yet. Nevertheless, Hernañdez-Jover *et al.* (1996) have been proposed an standard index called biogenic amine index (BAI), which is a sum quantity of cadaverine, putricine, tyramine and histamine for measuring the degree of BAs contamination in meat products i.e. BAI below 5mg/kg for fresh meat, 5-20mg/kg for acceptable meat, 20-50 mg/kg for low quality meat and above 50mg/kg for spoiled meat. Likewise; from a good manufacturing practice (GMP) point of view, a range of 50-100 ppm for histamine, 100-800 ppm for tyramine and 30 ppm for â-phenylethylamine or a sum of 100-200mg/kg total amine in any food is regarded as an acceptable level (Nout, 1994).

Conclusions

Biogenic amines are toxic compounds and found in several foods and beverages including meat and meat products. However, the toxic threshold for individual amine is difficult to establish due to alcohol, amine oxidase inhibiting drugs and some other amine such as cadaverine that may act as a potentiator to histamine food poisoning. Normally, a healthy individual can metabolize BAs to physiologically less active compounds by special enzyme system called monoamine oxidase (MOA) and diamine oxidase (DOA), though at high intake of such amines through muscle foods can interfere the catabolism of enzyme systems caused by several toxicological effects on human health. In addition, some species of bacteria like *Micrococci* and *Staphylococci* also have amine oxidase activity. Hence, the use of such amine oxidase positive starter culture in meat fermentation would have properties to control the BAs in muscle foods.

The accumulation of BAs in meat and meat products greatly depend on several factors such as pH, degree of proteolysis, ingredients used in processing, bacterial strains, growth kinetic of spoilage bacteria as well as processing and storage conditions. In conclusion, amino acid decarboxylase bacterial contamination either in raw material or during processing or storage stages of products seems to be critical control points (CCPs) to control the formation of such amines in muscle foods. Therefore, good handling practices (GHP) as well as good manufacturing practice (GMP) practices must be implemented during processing of any muscle food product to produce a hygienic and safety product having minimum or within limit of amines that would facade no health risk to consumers.

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Latte (Amaranth): A Nutritious Alternative Grain as a Functional Food

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Amaranth, an under utilized exploited grain with promising nutritional value, has recently recognized as a potential functional food grain. Amaranth contains high level of protein and dietary fiber compared to commonly consume other cereal grains and is good source of some vitamins (such as Vitamin A, C, E and Vitamin B-groups) and dietary minerals (scuh as Mg, Fe and Zn). Along with its nutritional superiority, amaranth has also established as a functional food having positive influences in the prevention and control of several human diseases especially for cardiovascular disease and diabetes. This potential grain has a wide variety of applications in household as well as in the food industries. This article reviews the nutritional and health promotional functional properties of amaranth grains.

Keywords: Amaranth, Nutritional value, Functional foods.

Introduction

The name amaranth (locally called as '*Latte*' in Nepali) comes from the Greek for "never-fading flower". Amaranth is a bushy plant that grows 5 to 7 feet, with broad leaves and a showy flower head of small, red or magenta, clover like flowers which are profuse, and constitute the plants exquisite, feathery plumes. The seed heads resemble corn tassels, but are somewhat bushier. The seeds are tiny (1/32"); a lens shaped, and is a golden to creamy tan color and is about the size of poppy seeds.

Not a true cereal grain, amaranth is sometimes called a 'pseudo-grain' and has been referred to as a herb or even a vegetable. There are 60 species of amaranth and both grain amaranth and leaves are utilized for use for human as well as animal foods (Martirosyan *et al.*, 2007). Although amaranth has been produced in different countries for centuries, only recently their remarkable nutritional properties have been recognized. Many researches have indicated that amaranth is an extremely nutritious grain therefore its popularity is growing in both developed and developing countries.

This paper provides a current overview on grain amaranth with an emphasis on currently available information about the nutritional superiority compared to other common grains to promote more efficient production and utilization of this under-utilized crop.

Proximate composition, food and nutritional value of amaranth seed

The nutritional value of amaranth has been extensively studied. An average proximate composition of amaranth seed is shown in Table 1. The seed contains about 90 percent dry matter. On an average, the protein, fat, dietary fiber and ash content are 13.0, 5.9, 13.6 and 2.7%, respectively. Amaranth seed is higher in protein than in most common grains except soybeans and just 150 grams of the grain is all that's required to supply an adult with 100% of the daily requirement of protein. Amaranth is good source of vegetable proteins originally characterized by the best indices of equilibration in amino acid composition as compared with the proteins of grains and legumes. The protein of amaranth has high content of water and salt soluble fractions, which contribute to a more complete assimilation of a product by human gut microflora.

Table 1. Proximate composition (average) of amaranth Seed

Parameters	*Values (g/100 g FW)*
Moisture	9.8
Dry matter	90.2
Protein	13.0
Fat	5.9
Dietary fiber	13.6
Ash	2.7
Carbohydrate	68.6
Energy (Kcal)	374

Source: Teutonico & Knorr, 1985.

The potential complimentary nature of amaranth protein has been studied by combining amaranth with wheat (Pant, 1985), sorghum (Pedersen *et al.*,1987) and maize (Tovar & Carpenter, 1982; Sanchez-Marroquin *et al.*, 1985). Ordinary maize meal supplemented with as little as 12.7% (by weight) of toasted amaranth flour provides a nutritionally superior source of protein that can satisfy a good portion of the protein requirement of young children, and provide approximately 70% of diet energy (Morales *et al.*, 1988). Amaranth protein has well balance of essential amino acids (Table 2) and compares well with the FAO/WHO protein standard indicating its high biological value and nutritional superiority. Amaranth proteins contain a balanced composition of essential amino acids such as lysine, mehionine and others, of which on or two are often low in beans wheat, and other common cereals. Hence, amaranth can become a valuable addition to wheat

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and cereals grain to improve the protein quality of the products. A combination of rice and amaranth in a 1:1 ratio has been reported to approach the FAO/WHO protein specifications (Singhal & Kulkami, 1988).

Amaranth fat/lipid is considered relatively good fat because of its composition. The characteristics of amaranth fat are shown in Table 3. Amaranth fat containing biologically active compounds such as squalen, tocopherol, phospholipids and phytosterols, can be used for food and medical purpose. Amaranth fat contains large amounts of squalen and tocopherol (Table 3), which are known to positively affect the biosynthesis of cholesterol. Owning to this principle several researchers has reported that amaranth oil can lower the plasma cholesterol content (Berger *et al.*, 2003; Konyk *et al.*, 2002). Amaranth fat has high level of unsaturation (about 75%), containing almost 50% linoleic acid (Table 4), which is necessary for human nutrition. The ratio of saturated and unsaturated fatty acids is approximately 1:3 (Pogojeva *et al.*, 2006). Therefore, the inclusion of amaranth in the diet may contribute to an increase in the concentration of polyunsaturated fatty acids, particularly long-chain acids of omega 3 families. Studies have suggested that a reduction in the consumption of saturated fatty acids and an increase in unsaturated fatty acids are beneficial and prevent cardiovascular disease (CVD). Therefore consumption of amaranth and amaranth oil may be helpful to CVD patients. The influence of amaranth fat upon lipid metabolism has been studied by Miroshnichenko *et al.* (2005) and reported that amaranth fat positively contributes to the cholesterol reduction in the blood of tested animals without change of its level in liver. Similarly, a case study conducted by Davidson (2005) on the effect of amaranth seed (oil) in the prevention of heart disease revealed that grain amaranth with closer supervision and monitoring, the coronary heart diseases can be reduced tremendously or controlled.

Table 2: Essential amino acid composition of amaranth grain

Essential amino acids (g/100g)	*Amaranth*	*Wheat*	*Soybean*	*FAO/WHO standard*
Lysine	5.95	0.23	2.30	5.40
Leucine	4.20	0.71	2.80	7.0
Isoleucine	2.71	0.36	1.67	4.0
Phenylalanine	4.70	0.52	1.80	6.00
Methionine	0.64	0.18	0.45	3.50
Threonine	3.25	0.28	1.50	4.00
Tryptophan	1.82	0.13	0.50	1.00
Valine	3.85	0.42	1.70	5.00

(Source: Davidson, 2005.)

Table 3: Characteristics of amaranth fat/lipid

Parameters	*Values*
Fractional composition of lipid (%)	
Triglycerides	79
Phytosterols	2
Free fatty acid	4
Phospholipids	7
Squalen	6
Sterol ester	2
Tocopherol (mg/100g)	200
Carotenoid (mg/100g)	0.45
B- Carotene (mg/100g)	0.05
Lutein (mg/100g)	0.4
Acid number (mg KOH/g)	2.5
Peroxide value (mol/kg)	3.4
Iodine number	70

(Source: Miroshnichenk *et al.*, 2005.)

Table 4: Fatty acid composition of amaranth fat (Lipid)

Fatty acids (Shorthand designation)	*Mass fraction of fatty acids (%)*
14:0	0.14
15:0	0.15
16:0	19.19
16:1	0.13
17:0	1.05
18:0	3.38
18:1	22.64
18:2	49.89
18:3 ?6	0.35
18:3 ?3	1.o1
20:0	0.16
20:1	1.04
22:0	0.32
22:1	0.07
24:0	0.05
24:1	0.43

(Sources: Pogojeva *et al.*, 2006.)

Amaranth is one of the highest grains in fiber content. Food and Nutrition Board, Institute of Medicine, USA recommended approximately 14g / 1000 Kcal as a RDA for dietary fiber (FNB, 2007). This means 100 g of amaranth nearly meets 50% of the RDA for a healthy human. The high dietary fiber content makes amaranth an effective agent for good intestinal functioning and effective against several health complications including cancers, cardiovascular disease, diabetes, obesity etc.(Malhotra & Bala, 2006). Evidence relating the total dietary fiber content of the diet to the incidence of several colonic and metabolic diseases is epidemiological in nature (Burkitt & Trowell, 1975). Since the role of dietary fiber in the diet as a preventive agent for problems of the alimentary and vascular system is well documented (Malhotra & Bala, 2006) consumption of amaranth can provide good source of dietary fiber for good health.

Amaranth is good source of dietary vitamins such as Vitamin A, C, E and Vitamin B-groups (Table 5). The data presented in Table 5 shows that the occurrences of these vitamins are in higher amounts in amaranth seed compared to that of common cereal grains. This implies that amaranth seed could be a good substitute for people suffering from these common vitamin deficiencies, especially vitamin A, which is a very big problem in many developing countries. Amaranth is also good source of dietary minerals, especially Magnesium, Iron, Zinc, and comparatively contains higher mineral content then common cereal grains (Table 6).

Table 5: Vitamins content (per 100 g) in amaranth grain and common cereals

Vitamins	*Amaranth*	*Maize*	*Wheat*	*Soybean*	*Rice*	*Finger millet*	*Sorghum*	*FAO/WHO RDA*
Vit. A (IU)	6100	-	-	-	-	-	21	5000
Thiamine, B1 (mg)	1.29	0.43	0.54	0.73	0.08	0.24	0.37	1.40
Riboflavin, B2 (mg)	2.10	0.22	0.12	0.39	0.06	0.11	0.14	1.70
Niacin , B3 (mg)	8.40	4.10	6.36	3.20	1.80	1.00	2.80	18.00
Pyrodoxine, B6 (mg)	1.22	0.58	0.02	-	0.02	-	0.50	1.50
Folic acid (μg)	49.00	-	-	-	9.10	-	-	400
Vit. C (mg)	4.63	-	-	-	-	1.00	-	45
Vit. E (mg)	1.03	0.25	0.37	0.01	0.13	0.21	-	10
Biotin (μg)	51.00	-	-	-	-	-	7.00	30.0
Pantothenic, B5 (mg)	1.15	0.47	-	-	-	-	1.00	5.0

(Source: Davidson, 2006; and WHO data base, 2004)

Table 6: Mineral composition (mg/100g) of amaranth and some common grains

Minerals	*Amaranth*	*Maize*	*Wheat*	*Soybean*	*Rice*	*Finger millet*	*Sorghum*	*FAO/WHO RDA*
Phosphorous	570	234	383	690	130	285	368	700
Potassium	532	320	-	-	130	195	220	-
Calcium	217	8	39	240	32	8	21	1300
Magnesium	319	142	288	-	130	114	140	260
Iron	21	3	3.5	11.5	0.9	3	507	27.4
Copper	0.86	0.35	0.9	P	0.25	0.5	1.8	-
Manganese	2.9	0.55	-	-	1.1	1.9	-	2.3
Zinc	3.4	2.5	1.0	-	1.2	1.5	-	4.5
Sodium	22	39	3	-	6	5	19	-

Source: Davidson, 2006.

Use of amaranth as food

Amaranth can be used as a whole grain in food preparations. It can also be puffed (like tiny popped corn) or ground into whole grain flour. Amaranth can be cooked as a cereal, ground into flour, popped like popcorn, sprouted, or toasted. Similarly, amaranth can be used in a number of food products including breakfast cereals, confectionery products, salad condiments, baked products (breads, muffins, cookies, and biscuits), extruded snack foods and chips, pastas, health foods, soups, and dietetic products. Because of the unique characteristic and nutritional profile of the seed, amaranth has been identified as a new potential crop for certain specialized applications in health foods and baked goods.

Amaranth: as a functional food grains

Amaranth, along with its nutritional superiority, has also established as a functional foods having positive influences in the prevention and control of several human diseases especially for cardiovascular disease and diabetes. In accordance to the studies conducted at the faculty of Medicine, Charles University Parge, University Hospital in the Czech Republic, foods containing whole amaranth provide

substrate that are predictable in reducing disease risks, especially prevention of cardiovascular disease (Zadak & Jelinek, 2001). A case study conducted in Kenya (Davidson , 2005) reported that consumption of foods containing amaranth can reduced or controlled the coronary heart disease. Pogojeva *et al.* (2006) has reported that the application of amaranth oil against the background of antiatherogenic diet in patients suffering from coronary heart disease and hypertension accompanied with obesity has more significant hypolipidemic action, the level of intensity of which is proportionate to the dose of amaranth oil, and recommended amaranth oil as a product for dietic nutrition in patients suffering from cardiovascular disease (atherosclerosis, coronal heart disease, hypertension, hyperlipoproteidemia, etc) and obesity. Recently, Kim *et al.* (2006) studied the antidiabetic and antioxidative effect of amaranth grain and amaranth oil in streptozotocin-induced diabetic rats and reported that both amaranth grain and amaranth oil supplement, as an antioxidant therapy, could be beneficial for correcting hyperglycemia and preventing diabetic complications (Table 7). Studied data indicated that serum glucose levels were about six-fold elevated in STZ- diabetic rats, and Amaranth grains (AG) and Amaranth oil (AO) supplementation significantly decreased STZ-induced hyperglycemia by 77 and 81%. Accordingly, there was a significant decrease in serum insulin concentrations in diabetic rats compared with normal controls, and administration of AG and AO tended to increase serum insulin levels.

Table 7: Effect of amaranth grain and amaranth oil on food intake, body weight, serum glucose, insulin, GPT and GOT concentration

Parameters	*Normal control*	*Diabetic control*	*Diabetic-AG*	*Diabetic-AO*
Food intake (g /day)	15.4	19.7	14.9	12.0
Body weight gain (g/day)	3.0	-4.0	-2.9	-3.7
Serum				
Fasting glucose (mg/dl)	87.3	496.5	164.4	145.1
Insulin (μU/ml)	21.3	9.8	14.9	16.1
GPT (U/ml)	25.2	52.0	33.1	49.9
GOT (U/ml)	64.6	113.7	62.8	48.4

(Adopted from: Kim *et al.* 2006. AG: amaranth grain; and AO: amaranth oil.)

Conclusions

The food and nutritional value, functional properties, current, and future applications of amaranths demonstrate the food potential of this underutilized crop. Additionally *in vitro* and *in vivo* studies have revealed the health promotional activities of amaranth, especially in the prevention of chronic disease like CVD and diabetes. Therefore, this underutilized grain could be a very potential health food or more importantly can serve as a new natural functional food, which may find wide application in both household and industrial uses. However, in the food processing area, research and development work is needed on the functionality of grain amaranth and the effects of processing on functionality and nutritional quality of amaranth. The main challenge for R&D is to incorporate amaranth into existing food formulations to modify their functional and nutritional quality, as well as to create entirely new products from amaranth.

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Functional Properties and Food Applications of *Chiraito* – A Medicinal Plant of Nepal

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Chiraito (Swertia chirayita) is a medicinal plant indigenous to temperate Himalaya. Its medicinal usage is reported in Nepalese Medicinal Plants, Indian Pharmaceutical codes, the British and American pharmacopoeias and in different traditional systems of medicines such as Ayurveda, Unani and Siddha. The plant is bitter tonic and is used in treatment of fever and curing various skin diseases. S. chirayita has an established domestic and international market, which is increasing at a rate of 10% annually.This article briefly reviews the available information on different aspects of S. chirayita with regard to its probable application in food.

Keywords: Bitter, *Swertia chirayita*, Nepal, Medicinal Plant, Functional Food.

Introduction

Nepal, a small Himalayan country, is located between latitude 26°22' and 30°27' north and longitude 80°04' and 88°12' east, with area of 147,181 km^2 and population of 23 million (growth rate 2.1%) (GoN, 2002). Climatically, Nepal lies in the temperate zone with an added advantage of altitude from 1220 to 8848 m, except for a few places that lie below 1220 m. Due to its geographic and climatic diversity, the relatively small country of Nepal, has significantly diverse ecosystems (Chaudhary, 1998; Subedi, 1999), producing a wide range of unique and valuable medicinal plant resources. Representing only 0.01% of earth's land area, Nepal is gifted by nature with 2.6% of all flowering plants, 9.3% birds and 4.5% of mammals of the world. Out of an estimated 9000 species found in the eastern Himalaya as a whole, 39% are endemic to this mountain range Nearly 7,000 species of higher plants are found in Nepal, of which 5% are endemic to Nepal and 10% are medicinal and aromatic plants. With 75 vegetation types ranging from dense tropical forests to alpine vegetation that covers over 50% of the total geographical area of the country forms the land resource base for the provision of medicinal plants (Myers, 1988; Myers, 1990).

As such, Nepal is very rich in biological resources, including native medicinal plants. The use of plants as medicine is widespread throughout the world. In many areas of rural Nepal, traditional knowledge and practices are passed down entirely through the oral tradition and personal experience and as trade secrets in families of certain communities, a practice protected tradition (Dahal *et al.*, 2005).

Bitter herbs such as *chiraito* (*Swertia chirayita*) have been used, for generations by the Himalayan villagers of Nepal, to reduce fever, to improve appetite and to eliminate poisons from the body. The whole plant is intensely bitter in taste (Nadkarni, 1998) and used in various treatments, for example, blood purifier, skin disease, bitter tonic for fever, indigestion, laxative, anthelmentic, antidiarrhoetic, antiperiodic and tonic for patients suffering from gout or bronchial asthma (Mukherjee, 1953; Dastur, 1956; Anon., 1976; Dey, 1980; Kirtikar & Basu, 1984). The bitter principle and water extract of *Chiraito* collectd from various ecological regions of Nepal ranges from 1.21% to 9% , and 8% to 12.4% respectively (DoPR, 2005b). The greatest amount of bitter principle was found in wild variety of Terathum district of eastern Nepal (DoPR, 2005b).

It is possible to use *Chiraito* to develop appropriate functional foods in powder, tablet and beverages forms and evaluate their efficacy in terms of enhancing appetite and digestive food supplements along with other useful functional foods for remedy of fever, jaundice, etc. This article wishes to explore the available information on probable use of *Chiraito* for food application.

Botanical aspects of *chiraito*

Swertia chirayita (Roxb. ex Fleming) H. Karst. is also mentioned in the literature as *Swertia chirata*, Buch.-Ham.; *Ophelia chirata* Grisebach.; *Agathotes chirayita* Don.; *Gentiana chirayita* Roxburgh (Clarke, 1885; Anon., 1982; Kirtikar & Basu, 1984; Duke, 2002) and *Gentiana floribunda* Don(Clarke,1885). It is known by an array of names, suggesting its widespread use. *Chiraito* is called Chirraito, Chiraita, Tite, pothi chiraito, *(Nepali)*, khalu *(Newari)*, khupli *(Rai)*, lektite *(Doteli)*, Kiratatikta *(Sanskrit)*,Tenko *(Gurung)*, Sungkhinwa *(Limbu)* (all these terms within brackets are languages ethnic to Nepal) in Nepal (Bhattarai, 1998), Anaryatikta, Ardhatikta, Bhunimba, Chiratika, Chiratitka, Haima, Jvarantaka, Kairata, Kandatiktaka, Kiranta, Kirataka, Kirata Tikta, Naditikta, Naipala, Nepalanimba, chiretta (English), Nidrari, Ramasenka, Sannipatha, Sutiktaka, Trinanimba, and Viktaka (Anon., 1982; Kirtikar & Basu, 1984) in Sanskrit, Cherayata in Patna, Chiraita and Kiraita in Mumbai, Chirayatin in Gujarat, Chireta in Bengal, Nilaveppa in Kerala, and Sekhagi in Burma. It is also called Chiaravata (Urdu); Qasabuzzarirah (Arab, Farsi); Charayatah (Deccan); Nelabevu (Kannada); Nenilawandi, Nilavembu, Shirattakuchi (Tamil). The trade name of *S. chirayita* is chiretta (Anon., 1982; Kirtikar & Basu, 1984)

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The plant is a native of temperate Himalayas, found at an altitude of 1200–3000 m (4000 to 10,000 ft), from Kashmir to Bhutan, and in the Khasi hills at 1200–1500 m (4000 to 5000 ft)(Clarke,1885; Anon.,1982). It can be grown in sub-temperate regions between 1500 and 2100 m altitudes (Bentley & Trimen, 1880). The genus *Swertia* Linn. consists of annual and perennial herbs. There is no consistency in the literature citing the habit of *Swertia chirayita*. Some authors have described *chiraito* as an annual (Anon., 1982; Kirtikar & Basu, 1984) and others as biennial or pluri-annual (Edwards, 1993). It is not clear whether the plant behaves differently due to climatic conditions or varying genotypes. The plant can be grown in a variety of soils with sandy loam rich in carbon and humus. It is also found in open ground and recently slash-and-burnt forests (Edwards, 1993). *S. chirayita* has an erect, about 2–3 ft long stem, the middle portion is round, while the upper is four-angled, with a prominent decurrent line at each angle. The stems are orange brown (Anon.,1982) or purplish in colour (Bentley & Trimen, 1880), and contain large continuous yellowish pith. The root is simple, tapering and stout, short, almost 7 cm long and usually half an inch thick (Clarke, 1885; Bentley & Trimen, 1880).

Cytological work done on the species is poor (Joshi & Dhawan, 2005). Khoshoo & Tandon 1963, used pollen-mother cells for cytological studies in some Himalayan species of *Swertia*. The authors counted thirteen bivalents at metaphase I, and observed that one of them was bigger than the rest.

Flowering in *S. chirayita* is in the form of numerous small, axillary, opposite, lax cymes arranged as short branches and the whole inflorescence is 2 ft long. Flowers are small, stalked, green-yellow, tinged with purple colour, rotate and tetramerous (Bentley & Trimen, 1880; Kirtikar & Basu, 1984). The corolla is twice as long as the calyx and divided near the base into four ovate–lanceolate segments. The upper surface of the petal has a pair of nectaries covered with oblong scales and ending as fringes (Bentley & Trimen,1880). Fruit is a small, one-celled capsule with a transparent yellowish pericarp. It dehisces from above, septicidally into two valves. Seeds are numerous, minute, many-sided and angular. Floral characteristics such as colourful corolla and presence of nectaries support crosspollination in the species. Generally, bees (Apoidae, Hymenoptera) are the pollinators of *S. chirayita* (Khoshoo & Tandon 1963).

The plant is harvested for the drug industry when it sets into flowering in July–September (Bentley & Trimen,1880; Anon.,1982). Seed setting commences around October–November and seeds germinate immediately after shedding. Only a few scattered reports in the literature suggest germination studies and nursery practices of *S. chirata* (Raina *et. al.*, 1994; Basnet, 2001). Ninety-one per cent seed germination was reported after 3°C chilling treatment for fifteen days (Raina *et. al.*, 1994), whereas another study reported a maximum of 81% germination (Basnet, 2001). An observation at the post-germination growth stage revealed that *S. chirata* is a slow-growing species (Basnet, 2001). Low germination percentage and viability of the seeds, long gestation periods and delicate field-handling are some of the factors which discourage commercial cultivation of the plant (Badola & Pal, 2002).

Functional characteristics of *chiraito*

S. chirayita belongs to family Gentianaceae, which records the occurrence of taxonomically informative molecules, namely iridoids, xanthones, mangiferin and C-glucoflavones. Reviews detailing the chemical constituents of the *Swertia* genus have been reported (Wang & Yang, 1992; Rahman & Arfan, 1997; Pant *et al.*, 2000). A number of workers have shown that the plant contained bitter glucosidal components, chiratin and amarogentin that may protect the liver against carbon tetra chloride poisoning (Harding, 1989; Chakravarty *et. al.*, 1991). The widespread uses of *S. chirayita* in traditional medicines have resulted in considerable chemical analysis of the plant, and active principles which attribute the plant its medicinal properties have been identified (Joshi & Dhawan, 2005) and isolated (Table 1).

The plant is gathered during the late stages of flowering, commonly tied up in flattish bundles about 3 ft long and 1.5 to 2 lbs in weight (Bentley & Trimen,1880) and is sold in the market as dried brownish stems with root and leaves intact. *S. chirayita* is used in British and American pharmacopoeias as tinctures and infusions. According to Ayurvedic pharmacology (Joshi, 2000), *chiraito* is described as bitter in taste (*rasa*). The thermal action (*virya*) of *chiraito* is defined as cooling (*shita*). *Chiraito* is light (*laghu*), i.e. easily digestible, and *ruksha* (*dry*). These characteristics drain heat from the blood and liver. Its use has also been mentioned in Unani medicine (Mukherji, 1953). Concoction of *chiraito* with cardamom, turmeric and kutki is given for gastrointestinal infections, and along with ginger it is considered good for fever (Kirtikar & Basu, 1984). When given along with neem, manjishta and gotu kola, it serves as a cure for various skin problems. It is used in combination with other drugs in cases of scorpion bite (Nandkarni, 1976).

At least five different medicines are being made in *Singhadurbar Vaidhya Khana* (under Minsitry of Health, Government of Nepal) including *Rohitkayadi Churna* (powder for the treatment of jaundice) and *Pitdhan vati* (tablet for the treatment of headache and fever) (Personal Communication, 2007).

Probable application of *Chiraito* in food

Various food applications of *chiraito* have been reported. To treat diabetes, the rural Nepalese people use herbal treatments either alone or in combination with other form of treatment or with other food bases (Bhandari *et al.*, 2006). Studies have shown that some non-traditional Nepalese herbal plants contained significant antidiabetic activity and suggest that these herbs and food plants could have their applicability as

Table 1: Biological activities attributed to *S. Chirayita*

Activity	*Reference*
Alternative	Duke, 2002;
Antihelmintic	Ray *et al.*, 1996
Antileishmaniak	Medda *et al.*, 1999
Anticholinergic	Rafatulla *et al.*, 1993
Anticonvulsant	Bhattacharya *et al.*, 1976
Antiedemic	Blaschek *et al.*, 1998
Antiinflammatory	Korte, 1955; Islam, 1995, Kumar *et al.*, 2002; Banerjee *et al.*, 2002
Antimalarial	Goyal, 1981
Antipyretic	Blaschek *et al.*, 1998
Antitubercular	Kamatsu *et al.*, 1971
Bitter	Dalal & Shah, 1956
Cardio stimulant	Blaschek *et al.*, 1998
Cholagogue	Kirtikar & Basu, 1984
Choleretic	Blaschek *et al.*, 1998
CNS depressant	Ghoshal *et al.*, 1973
Emollient	Duke, 2002; Sharma, 1982
Hepatoprotective	Mukherjee *et al.*, 1997
Hypnotic	Duke, 2002; Ray *et al.*, 1996
Hypoglycemic/antidiabetic	Mukherjee & Mukherjee, 1978; Bajpai *et al.*, 1991; Chandrashekar *et al.*, 1990; Saxena & Mukherjee 1992; Saxena *et al.*, 1993
Laxative	Duke, 2002; Ray *et al.*, 1996
Secretagogue	Kirtikar & Basu, 1984
Stomachic	Nandkarni, 1976
Tonic	Nandkarni, 1976
Undersedative	Kirtikar & Basu, 1984
Vermifuge	Kirtikar & Basu, 1984

important antidiabetic agents and be used as food (Bhandari *et al.*, 2006).

Soliman & Badeaa (2006) tested essential oils of 12 medicinal plants for inhibitory activity against *Aspergillus flavus, A. parasiticus, A. ochraceus* and *Fusarium moniliforme.* The results indicate that the test toxigenic fungi are sensitive to the 12 essential oils, and particularly sensitive to thyme and cinnamon. The results also showed that the essential oils of thyme, cinnamon, anise and spearmint have more effect on fungal development and subsequent mycotoxin production in wheat grains. The other study regarding use of plant parts was done by Kim *et al.* (2006). Twenty-one plant parts were utilized as food in 42 different preparations. Approximately 82% of the plants studied had medicinal effects, with a wide range of efficacies (126 types). Of the medicinal plants, 52% were used for digestive problems, circulatory illnesses, and respiratory diseases. These results demonstrate that a high proportion of the food consumed in Korean temples is medicinal, and is used for a wide variety of diseases. There have been several studies on use of green tea in bread for increasing the functionality of bread (Personal communication, 2007). Studies on use of *Bergenia ciliata*, Haw. revealed the anti-diabetic potential of the fruit (Bhandari *et al.*, 2008). The study would help to develop medicinal preparations or nutraceutical and functional foods for diabetes and related symptoms (Bhandari *et al.*, 2008).

Thus it is possible to develop appropriate functional foods in powder, tablet and beverages forms and evaluate their efficacy in terms of enhancing appetite and digestive food supplements along with other useful functional foods for remedy of fever, jaundice, etc. The utilization aspects of all components of the plant would have to be explored as functional food and dietary supplements and identified for their potential benefits.

Trade of *chiraito* in Nepal

Some 104 non-timber forest products (NTFPs) items are commonly traded in Nepal. Among the high value NTFPs, *Swertia* species occupy one of the major positions in the trade (Table 2).

A total of nine species of *Swertia* are being traded in Nepal (DoPR, 2005a).

1. *Swertia chirayita* (Roxb. ex Fleming) H. Karst.
2. *Swertia angustifolia* Buch.-Ham. ex D. Don
3. *Swertia tetragona* Edgew
4. *Swertia racemosa* (Griseb.) C.B. Clarke
5. *Swertia ciliata* (D.Don ex G. Don) B.L. Burtt
6. *Swertia dilatata* C.B. Clarke
7. *Swertia multicaulis* D.Don
8. *Swertia alata* (Royale ex D. Don) C.B. Clarke
9. *Swertia nervosa* (G.Don) C.B. Clarke

Among these, *S. chirayita* plays a dominant role in trade covering about 80% of total traded volume of *chiraito* (*Swertia* spp.), and is superior in quality to other species. Most of the *chiraito* (80-90%) are exported as a crude drug to India, occasionally to China, Malaysia, Singapore, Germany, Italy, France, Switzerland, Sri Lanka, Bangladesh, Pakistan, or USA. Nepal trades about 50 percent of the world's total volume of *chiraito* (DoPR, 2005a). The market price of *chiraito* bears a frequent fluctuation as seen in the comparison of current (NRs* 80-150/Kg) and previous prices (NRs 400/Kg, in 2000) *[1 US dollar= 74.64 Nepalese currency (NRs), 2004]. The royalty rate is NRs 3.00/Kg for entire plant (*Hamro Ban*, 2003).

The widespread use of *S. chirayita* in traditional medicine reflects its pharmacological importance. However, existing populations of *S. chirayita* are diminishing. Hence according

Table 2: Trade of *Chiriato* in five developmental regions of Nepal (in Kg)

Fiscal Year	*Developmental Regions*					*Total Trade*
	Eastern	*Central*	*Western*	*Midwestern*	*Farwestern*	
1998/99	82789	66606	6464	4200	6392	166451
1999/2000	64387	62834	14216	24415	3470	169361
2000/01	111970	178650	17215	14481	15181	337497
2001/02	100410	36977	14827	25143	11057	188415

(Source : Hamro ban, 2003)

to the new International Union for Conservation of Nature and Natural Resources (IUCN) criteria *S. chirayita* has been categorized as critically endangered (Anon, 1997). This leads to a need for conservation of the plant. *S. chirayita* has been prioritized by the Department of Plant Resources (DoPR), Government of Nepal, for agriculture research technology development (DoPR, 2005b). About 29 different species of *chiraito* have been found in Nepal Among them, *Swertia graciliescens* is endemic to Nepal (Basnet, 2001).

Many farmers in Nepal have cultivated *chiraito* on their private lands because of good market price and high demand (DoPR, 2005b). The plant has a huge demand in the medicinal market and is an important factor for the economy of Nepal. About 45% of *chirayita* in the Himalayan region is collected from Nepal (Shah,1999). The trade and economics of *chiraito* is also affected by adulterants of the herb. *Andrographis paniculata* (green *chiraito*) (Girach *et al.*,1994), *Exacum etragonum* Roxb., *E. bicolor* Roxb., *E. pedunculatum* Linn., *Slevolgia orientalis* Griesb., *Swertiaalata* Royle., *S. angustifolia* Buch.-Ham., *S. bimaculata* Hook. f. and Thoms., *S. ciliata* G. Don, *S. densifolia* Greisb., *S. elegans* Wight., *S. lawii* Burkill., *S. minor* Griesb., *S. paniculata* Wall., *S. multiflora* Dalzell., are adulterants found along with true *chiraito* (Anon., 1982). *S. minor* Griesb. is used as a substitute for *chiraito* in treatment of malaria and other fevers (Anon.,1982).

However, substitutes such as *S. angustifolia* Buch.-Ham. and *S. alata* Royle. are inferior to *S. chirayita* in terms of bitterness (Anon.,1982). Karan *et al.* (1997) and Bhatia *et al.* (2003) reported comparative evaluation of morphological characters and chromatographic fingerprint profile for xanthones and secoiridoid bitters of *S. chirayita* along with other species of *Swertia*. The true *chiraito* can be distinguished from other substitutes and adulterants by its intense bitterness, brownish-purple stem (dark colour), continuous yellowish pith and petals with double nectaries.

There is a rapid loss of traditional medical knowledge and practices due to their dependency on verbal transformation, impacts of modern cultural transformation, and rapid land degradation (Manandhar, 1990a & 1990b; Joshi & Joshi, 2000). At the same time there is a depletion of resource bases due to over exploitation and lack of management systems (Edwards, 1993; Edwards, 1996; Subedi, 1999). Many indigenous species of medicinal plants have been lost in abyss in Nepal due to ignorance and lack of utilization. Many precious medicinals have reached the point of extinction due to poaching, illegal trading and lack of knowledge and awareness. The method of wild harvesting trading of raw materials without value addition and illegal export via small traders to India has limited economic benefits to the ethnic community of Nepal (Subedi, 1999).

The result is that the poor become poorer and end up destroying their only livelihood – the biodiversity rich forest. Though some of these herbs were used for the treatment of diabetes and traditionally produced some herbal medicines but there are no scientific grounds to prove it.

Concluding remarks

In many developing countries like Nepal, traditional medicine, in particular herbal medicine is sometimes the only affordable source for healthcare (Bhattarai, 1993; Manandhar, 1995; Shrestha & Joshi, 1993). As for the developed countries, the use of herbal medicine by the suffers of chronic disease is encouraged because there is concern about the adverse effects of chemical drugs and treatment using medicines of natural origin appears to offer more gentle means of managing such disease (Hamdan & Afifi, 2004; Klepser & Klepser, 1999; WHO, 2002).

Chiraito is one of the very effective medicinal plants which are used in traditional healing practices of the Himalayan regions of Nepal. It is very amazing that the whole plant is useful as medicine and been used for curing arrays of health implications and diseases. A propos to its ethno botanical aspect the plant is studied for functional phytochemicals known to date and also for elucidation of new active components with new frontiers for Indian and Chinese species. However, species indigenous to Nepal has not been studied in detail despite of expectation of more promising results as being inhabitant of very diverse and unique ecological zone of the world.

There is a wide scope for exploring different aspects of *S. chirayita.* Discrepancies remain about the habit of the plant. There are no established agro-techniques for promoting its

cultivation. Only preliminary studies have been done to provide information regarding germination and viability of *chiraito* seeds. The threatened status of the herb calls for establishing sustainable harvesting methods for *S. chirayita*. There are, of course, no established varieties or lines of *chiraito*. A strong need is felt to screen the different chemotypes of *chiraito* growing at different phyto-geographical locations. Similarly, biodiversity studies at morphological, biochemical and genetic levels will enable the research community to realize the extent of variability within the existing germplasm of *S. chirayita* and hence help in conservation of the plant.

Indigenous species of Nepal *S. chirayito* is not studied for the functional phytochemicals and its potential benefits in detail. Considering the range of different niches occupied by the plant, there is a possibility that many ecotypes and/or chemotypes of *S. chirayita* exist. It would be interesting to study the morphological, molecular and biochemical variations among different populations for *S. chirayita* and their use as functional food.

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Sources and Causes of Bacterial Load in Raw Milk

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Milk is the nutrient fluid secreted by mammary gland of female mammals.Raw milk as it leaves the udder of healthy cows, normally contains very low numbers of microorganisms and gradually increases which affects the shelf life of milk. Microbial contamination of raw milk can occur from a variety of microorganisms, sources, and quality likewise shelf-life of pasteurized fluid milk is dependent on the quality of the raw milk and other ingredients used and an effective cleaning and sanitation practices. Therefore, determining the cause of bacterial defects is not always straight-forward. High bacterial counts can also result from a combination of factors (i.e. dirty equipments and marginal cooling etc.). So, health and hygiene of the animal, environment in which the animal is housed and milked, and the procedures used in cleaning and sanitizing the milking and storage equipments and storage temperature are all key influencing factors on the quality of milk.

Keywords: Milk, Quality, Contamination, Shelf life, Coliform, Microbial counts

Introduction

Milk is the nutrient fluid secreted by the mammary gland of female mammals. The female ability to secrete milk is one of the defining characteristics of mammals. It provides the primary source of nutrition for newly born babies before they are able to digest more diverse foods. The early lactation milk is known as colostrum, and carries the mother's antibodies to the baby. It can reduce the risk of many diseases in both the mother and baby.

The exact component of raw milk varies by species, but it contains significant amounts of unsaturated fat, protein and calcium. These amounts are not large in comparison to other foods rich in them, including coconuts, fish, etc., because milk is predominantly composed of water.

Milk is synthesized in specialized cells of the mammary gland and is virtually sterile when secreted into the alveoli of the udder (Tolle, 1980). Beyond this stage of milk production, microbial contamination can generally occur from three main sources (Bramley & McKinnon, 1990); from within the udder, from the exterior of the udder and from the surface of milk handling and storage equipments. The health and hygiene of the cow, the environment in which the cow is housed and milked, and the procedures used in cleaning and sanitizing the milking and storage equipment are all key factors that influences the level of microbial contamination of raw milk. Equally important are the temperature and length of time of storage which allow microbial contaminants to multiply and increase in numbers. All these factors will influence the total bacterial counts or Standard Plate Count (SPC) and the types of bacteria present in bulk raw milk. This article reviews about the different sources and causes responsable for bacterial load and shelflife of milk.

Quality of milk

Consumers purchase pasteurized fluid milk with the belief that they are taking home a wholesome, nutritious and good quality product. To the consumer, quality means that the product tastes good and that it keeps well in their home refrigerator or has a long "shelf-life." Shelf-life can be defined as "the period of time that a product can be kept under practical storage conditions and still retain acceptable quality." In the case of pasteurized fluid milk "practical storage conditions" means held under refrigeration or less than 7.2°C (45°F) while "acceptable quality" means that product flavor, odor and appearance are satisfactory to the consumer (no consumer complaints) and that the milk is safe to drink. In the western countries, most dairy products have a shelf-life of 14 days or more. Some plants are striving for 21 days. Achieving this goal requires stringent processing parameters, rigorous product handling procedures and extreme efficacy in cleaning and sanitation programs (Tolle, 1980).In this context, current consumption and marketing patterns of Nepalese products is not satisfactory.

Quality defects in pasteurized milk products are most often the result of microbial contamination, growth and spoilage. Microbial defects usually become evident in the finished product through shelf-life evaluations or consumer complaints. Though poor quality raw milk can result in defective products, post-pasteurization contamination with psychotropic spoilage bacteria is most detrimental. In most cases, product contamination is the result of insufficient cleaning and sanitation of the processing equipments and unhygienic environment. Product contamination may occur even when it appears that a well-designed sanitation and

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quality control program is in plant. In the absence of post-pasteurization, certain strains of microorganisms (i.e. *Bacillus* spp) that are capable of surviving pasteurization and growing under refrigeration (Thermoduric psychrotrophs) can eventually grow and cause spoilage, generally later in shelf-life (Palmer, 1980).

Milk product quality is generally determined by sensory, chemical and microbiological analyses. These analyses begain when the milk leaves the cows' udder to the final product is consumed. To determine shelf-life, fluid milk is most often held at marginal refrigeration temperatures of 6.1-7.2°C (43-45°F) and evaluated by sensory and/or microbiological testing after the desired number of days (i.e. sell-by date plus 2-5 days). Though some prefer to evaluate shelf-life at more ideal holding temperatures of less than 3.3°C (38°F), marginal refrigeration temperatures allow potential product defects and sanitation deficiencies to become more evident. Sensory analyses require that someone who is familiar with and is sensitive to milk off-flavors smell and/or taste of milk. This type of evaluation is somewhat subjective, as people differ in their ability to detect off-flavors. In this regard, microbiological analyses can lend more insight into potential quality defects in dairy products. When pasteurized milk shelf-life is reduced due to microbial growth, it is most often the responsibility of the processing plant quality control personnel to determine the cause and source of contamination (Fenlon *et al.*, 1995)

Microbial contamination from within the udder

Raw milk as it leaves the udder of healthy cows normally contains very low numbers of microorganisms and generally will contain less than 1000 total bacteria per mL (Kurweil, & Busse, 1973). In healthy cows, the teat cistern, teat canal, and the teat apex may be colonized by a variety of microorganisms though microbial contamination from within the udder of healthy animals is not considered to contribute significantly to the total numbers of microorganisms in the bulk milk, nor to the potential increase in bacterial numbers during refrigerated storage. Natural flora of the cow generally has little influence on SPC.

While the healthy udder should contribute very little to the total bacteria count of bulk milk, a cow with mastitis has the potential to shed large numbers of microorganisms into the milk supply. The influence of mastitis on the total bacteria count of bulk milk depends on the strain of infecting microorganism(s), the stage of infection and the percentage of the herd infected. Infected cows have the potential to shed in excess of 10^7 bacteria per mL. If the milk from one cow with 10^7 bacteria per mL comprises 1% of the bulk tank milk, the total bulk tank count, disregarding other sources, would be 10^5 per mL (Bramley & McKinnon, 1990).

Mastitis organisms found to most often influence the total bulk milk count are *Streptococcus* spp., most notably *S. agalactiae* and *S. uberis*, though other mastitis pathogens have the potential to influence the bulk tank count as well. *Staphylococcus aureus* is not considered to be a frequent contributor to total bulk tank counts though counts as high as 60,000/mL have been documented (Gonzalez *et al.*, 1986). Detection of implied pathogens does not necessarily indicate that they originated from cows with mastitis. Potential environmental mastitis pathogens and/or similar organisms can occur in milk as a result of other contributing factors such as dirty cows, poor equipment cleaning and/or poor cooling. An increase in SPC can sometimes serve as supportive evidence that a mastitis bacterium may have caused an increase in the bulk milk bacteria count. This seems to hold true more for *Streptococcus* spp. than for *S. aureus*, which appears to be shed into the milk in lower numbers (Fenlon *et al.*, 1995). Correlations of somatic cell responses and environmental mastitis organisms, including coliform bacteria, streptococci, and certain coagulase-negative *Staphylococcus* spp., were found to be poor as well. These organisms are by nature associated with the cow's environment and may influence bulk milk bacteria counts through other mean (Zehner *et al.*, 1986). *S. agalactiae* and *S. aureus* are not considered to grow significantly on soiled milking equipment or under conditions of marginal or poor cooling. Their presence in bulk tank milks is considered strong evidence that they originated from infected cows (Gonzalez *et al.*, 1986).

Microbial contamination from the exterior of udder

The exterior of the udder and teats can contribute microorganisms that are naturally associated with the skin of the animal as well as microorganisms that are derived from the environment in which the cow is housed and milked. In general, the direct influence of natural inhabitants as contaminants in the total bulk milk count is considered to be small and most of these organisms do not grow competitively in milk. Of more importance is the contribution of microorganisms from teats soiled with manure, mud, feeds or bedding.

Teats and udders of cows inevitably become soiled while they are lying in stalls or when allowed in muddy barnyards. Used bedding has been shown to harbor large numbers of microorganisms. Total counts often exceed 10^8-10^{10}/ g (Zehner *et al.*, 1986). Organisms associated with bedding materials that contaminate the surface of teats and udders include *Streptococci, Staphylococci*, spore-formers, coliforms and other Gram-negative bacteria. Both thermoduric (bacteria that survive pasteurization) and psychrotrophic (bacteria that grow under refrigeration) strains of bacteria are commonly found on teat (Bramley & McKinnon, 1990) indicating that contamination from the exterior of the udder.

The influence of dirty cow on total bacteria counts depends on the extent of soiling of the teat surface and the washing procedures used immediately before milking. For example, if 1 g of teat soil containing 10^8 bacteria is allowed into the milk of one cow giving approximately 30 lb. (~13,400 g) of milk, the total bacteria count for that cow's milk, excluding other sources, would be in excess of 7,000 per mL. Milking heavily soiled cows could potentially result in bulk milk counts exceeding 10^4 per mL. Several studies have investigated for pre-milking hygienic condition of udder related to the bacterial count of milk (Palmer, 1980). Generally, thorough cleaning of the teat with a sanitizing solution (spray, wet towel or dip) followed by thorough drying with a clean towel is effective to retard the number of microorganisms in milk. Counts of coliform bacteria, though highly associated with manure, barnyard mud, and used bedding, were relatively low in these studies, even for the untreated cows suggesting that higher coliform counts in bulk milk are more likely to occur due to other factors (i.e. equipments, mastitis condition etc.).

Influence of cleaning equipments and sanitizing procedures

The degree of cleanliness of the milking system probably influences the total bulk milk bacteria count as much, if not more than any other factor (Olson & Mocquat, 1980). Milk residue left on equipment contact surfaces supports the growth of a variety of microorganisms. Organisms considered being natural inhabitants of the teat canal; apex and skin are not thought to grow significantly on soiled milk contact surfaces or during refrigerated storage of milk. This generally holds true for organisms associated with contagious mastitis (i.e. *S. agalactiae*) though it is possible that certain strains associated with environmental mastitis (i.e. coliforms) may be able to grow to significant numbers. In general, environmental contaminants (i.e. from bedding, manure, feeds) are more likely to grow on soiled equipment. Water used on the farm might also be another source of microorganisms, especially psychrotrophs (Bramley & McKinnon, 1990).

Cleaning and sanitizing procedures can influence the degree and type of microbial growth on milk contact surfaces by leaving behind milk residues that support growth, as well as by setting up conditions that might select for specific microbial groups. More resistant and/or thermoduric bacteria may endure in low numbers on equipment surfaces that are considered to be efficiently cleaned with hot water. If milk residue is left behind (i.e. milk stone), growth of these types of organisms, may persist. Old cracked rubber parts are also associated with higher levels of thermoduric bacteria. Significant growth of these microorganisms to a point where they influence the total bulk tank count may take several days to weeks (Thomas *et al.*, 1966).

Less efficient cleaning, using lower temperatures and/or the absence of sanitizers tends to select for the faster growing, less resistant organisms, principally Gram-negative rods (coliforms and Pseudomonads) and lactic streptococci. Effective use of chlorine or iodine sanitizers has been associated with reduced levels of psychrotrophic bacteria that cause high PICs (Jackson & Clegg, 1965). Psychrotrophic bacteria tend to be present in higher count in milk and are often associated with occasional neglect of proper cleaning or poor sanitizing procedures (Olson & Mocquat, 1980; Thomas *et al.*, 1966) and/or poorly cleaned refrigerated bulk tanks (MacKenzie, 1973).

Milk storage temperature and time

Refrigeration storage, while preventing the growth of non-psychrotroph bacteria, will select for psychrotrophic microorganisms that enter the milk from soiled cows, dirty equipment and the environment. Minimizing the level of milk contamination from these sources will help prevent psychrotrophs from growing to significant levels in the bulk tank during the storage period on the farm or at the dairy plant. In general these organisms are not thermoduric and will not survive after pasteurization. The longer raw milk is held before processing, the greater the chance that psychrotrophs will increase in numbers. Holding milk near 7.2°C (45°F) allows much quicker growth than milk held below 4.4°C (40°F). Though milk produced under ideal conditions may have an initial psychrotroph population of less than 10% of the total bulk tank count, psychrotrophic bacteria can become the dominant microflora after 2-3 days at 4.4°C (40°F) (Gehringer, 1980). Colder temperatures of 1-2°C will delay this shift, though not indefinitely.

Under conditions of poor cooling with temperatures greater than 7.2°C (45°F), bacteria other than psychrotrophs are able to grow rapidly and can become predominant in raw milk. Though incidents of poor cooling still occur, this defect is not as common as when milk was held and transported in cans. Streptococci have historically been associated with poor cooling of milk, appearing as pairs or chains of cocci (spherical bacteria) on microscopic examination of milk smears (Atherton & Dodge, 1970). These bacteria will increase the acidity of milk. Certain strains are also responsible for a "malty defect" that is easily detected by its distinct odor. Storage temperatures greater than 15°C (60°F) tend to select for these types of contaminants (Gehringer, 1980). Likewise poor cooling conditions allow the growth of bacteria that normally will not grow in properly refrigerated milk; it will not prevent the growth of typical psychrotrophic strains. Types of bacteria that grow and become significant will depend on the initial microflora of the milk (Bramley & McKinnon, 1990).

According to De (2000), the optimal storage temperature for milk after collection and processing is 5°C, because at

Table 1: Effect of storage temperature on bacterial growth in milk

Milk held for 18 h at temperature (°C)	*Bacterial growth factor*
0	1.00
5	1.05
10	1.80
15	10.00
20	200.00
25	1,20,000.00

(Source: De, 2000)

this temperature, there is relatively no growth of microorganism even after 18 h of storage (Table 1). Where as after 5°C, there is significant increase in microbial population.

Microbiological analysis for quality of milk

Following are microbiological procedures that are used to evaluate pasteurized fluid milk quality and shelf-life.

Standard Plate Count (SPC)

According to Nepal Gazette (2000), standard limit is 50,000/g (in SMP). The Standard Plate Count is an estimate of the total number of aerobic bacteria present in a sample that are capable of growth on SPC media when incubated at 32°C (89.6°F) for 48 h. The theory behind the Standard Plate Count is that individual bacteria (or tight groups or "clumps" of bacteria) will multiply and grow on SPC media to form a visible, countable colony (i.e. a colony forming unit or CFU). Colonies counted are expressed as the number of CFU per mL.

Generally the SPC of freshly pasteurized milk is less than 500/mL. Most often this initial SPC represents those bacteria that survive pasteurization (*Thermoduric bacteria*), though gross contamination after pasteurization can also result in high counts. Initial counts higher than 1000 SPC/mL suggest a potential contamination problem either in the raw milk supply or within the processing equipment (Richardson, 1985)

SPC and Shelf-Life

SPC is often used to evaluate the shelf-life of milk, so to extend the shelf life; every effort should be directed towards minimizing this amount. As milk is held under refrigeration temperature, bacteria that have the ability to grow under these conditions will increase in numbers as reflected in the SPC. These types of bacteria are referred to as psychrotrophs and are defined as those bacteria capable of growth at temperatures at or less than 7°C (44.6°F). In general, reducing storage temperature will decline the growth of all bacteria. Refrigeration storage prevents the growth of non-psychrotrophic bacteria; growth is negligible or virtually stops at the freezing point, even for psychrotrophic bacteria. Most psychrotrophic bacteria that rapidly spoil milk do not survive pasteurization. If they are present in milk, generally occur as post-pasteurization contaminants due to less than adequate sanitation facilities.

The SPC of freshly pasteurized milk is not a good indication of the numbers of psychrotrophs present since most bacteria that survive pasteurization are not psychrotrophic. Though most psychrotrophic bacteria are detected in the SPC procedure, they are indistinguishable from non-psychrotrophic bacteria. While the initial SPC may be 500/mL, only one (or less) of these bacteria may be a psychrotroph (it only takes one psychrotrophic contaminant per container to eventually cause spoilage). The actual number of psychrotrophs present can be estimated by plating the milk sample using the SPC procedure and incubating for 10 days at 7°C (44.6°F) instead of 32°C (89.6°F). This is a lengthy procedure and is not routinely used by most. However in shelf-life, milk stored at 6.1-7.2°C (43-45°F), the presence of

Table 2: Growth of psychrotrophic and non-psychrotrophic

Days at 43°F	*Non-psychrotrophs/mL*	*Psychrotrophs/mL*	*SPC/mL*
Initial	500	1	501
1	500	10	510
2	500	100	600
3	500	1000	1500
4	500	10000	10500
5	500	100000	100500
6	500	1000000	1000500

(Sources: Meer *et al.*, 1991)

psychrotrophic bacteria will become evident by an increase in the SPC over time. Non-psychrotrophic bacteria, by definition will not grow at these temperatures and will become insignificant in the overall count (Table 2).

Generally, when the SPC exceeds 10 million cfu/mL, the product will become unacceptable due to flavor defects related to bacterial growth and metabolism. The extent and type of spoilage will depend on the strain(s) of psychrotrophic bacteria present. The key factors to prevent spoilage and extending the shelf-life of a product is to prevent post-pasteurization contamination (PPC), through a well designed quality assurance program as one psychrotroph per container of milk would be enough to cause whole milk spoilage (Richardson, 1985).

Coliform count

According to Nepal Gazette (2000), standard limit is nil /mL. Coliform bacteria are used as "indicators" of sanitation during the handling and processing of milk products. Certain coliform bacteria originate from the intestinal tracts while others are environmental contaminants. Normally coliforms are killed by pasteurization, thus when present in milk, are regarded as "indicators" of post-pasteurization contamination as a result of poor sanitation. Though the limit for pasteurized milk is 10/mL, the detection of any coliform bacteria suggests that some point in processing has been neglected in regard to effective cleaning/sanitation. This procedure is routinely used for fresh pasteurized milk though it can be used in shelf-life evaluations

as well. As a rule, the detection of any coliforms in pasteurized milk indicates the causative agent to shorten the shelf-life of milk (Richardson, 1985).

Psychrotroph and gram-negative bacteria count

Generally, psychrotrophic bacteria are the cause of most shelf-life problems in fluid milk. Most common psychrotrophic organisms implicated in the spoilage of fluid milk are gram-negative rods, primarily belonging to the genus *Pseudomonas*. As a rule, gram-negative bacteria in general do not survive in pasteurization. This is why coliform bacteria, which are one type of gram-negative bacteria, are used as indicators of poor hygiene and post-pasteurization contamination in pasteurized milk products. Detection of coliform suggests the possibility for concurrent contamination with psychrotrophs and/or potential pathogens. The psychrotroph count will detect gram-negative and gram-positive psychrotrophs, though gram-positive organisms rarely cause problems in pasteurized fluid milk. Crystal Violet Tetrazolium Agar (CVTA) selects for gram-negative bacteria, since contamination levels are generally very low, CVTA counts are most useful when used with stress tests.

Thermoduric psychrotrophs

Thermoduric bacteria are those that survive in pasteurization or other heat treatments. Most thermoduric bacteria are not psychrotrophic, though certain bacteria that survive in pasteurization are capable to grow at refrigeration temperature. Thermoduric psychrotrophs occasionally spoil milk in the absence of gram-negative post-pasteurization contamination. These types of organisms generally grow slower and/or begin growth later, causing problems later in shelf-life. The Laboratory Pasteurization Count (LPC) is often used to estimate the number of bacteria in a raw milk supply that will survive pasteurization. The LPC is performed by heating raw milk to 62.8°C (145°F) for 30 minutes before plating for the SPC. To detect thermoduric psychrotrophs, milk that is laboratory pasteurized is plated for psychrotrophic organisms (SPC procedure incubated at 7°C for 10 days) as well as for the SPC. Alternatively, the heated milk can be stored at 7°C for 10+ days and then plated for SPC. A significant increase over the initial LPC indicates the presence of thermoduric psychrotrophs. Some of the most common thermoduric psychrotrophs are spore-formers (e.g. *Bacillus* spp.), which are more heat resistant and may require higher heat to cause spore germination. An alternative to the LPC is to heat milk at 80°C (176°F) for 10 minutes followed by rapid cooling. The milk can then be plated for psychrotrophs or the milk itself can be stored under refrigeration for 10-17 days and then plated with the SPC. Any significant growth would indicate potential spoilage by spore-forming psychrotrophs. Heating larger sample volumes (i.e. 200 mL) for LPC or psychrotrophic spore counts and holding under refrigeration will help detect low-level contamination (Richardson, 1985).

Stress tests

In most cases, gram-negative psychrotrophic spoilage organisms contaminate product at very low levels, often less than 1 per mL, below the level of detection in most plating procedures. This still presents a major concern because one psychrotrophic bacterium with a doubling time of 6 h can spoil a quart (= 946 mL) of refrigerated milk in less than ten days (counts of greater than 10 million CFU per mL). The SPC procedure generally is not sensitive enough to detect low-level contamination in line-samples or finished product. The number of psychrotrophs may only be a small proportion of the total SPC and are indistinguishable from non-psychrotrophic bacteria with this procedure. Therefore, finding low-level of contamination is a difficult task. It generally requires a large sample size and an incubation period or "stress test" which selects for psychrotrophic organisms and allows them to increase to detectable levels (Richardson, 1985).

Conclusions

Raw milk as it leaves the udder of healthy animal normally contains very low numbers of microorganisms and gradually increases which affects the shelf life of milk. Microbial contamination of raw milk can occur from a variety of microorganisms, sources etc. Because of this, determining the cause of bacterial defects is not always straight forward. The quality and shelf-life of pasteurized fluid milk is dependent on the quality of the raw milk and other ingredients used and on an effective cleaning and sanitation practice as well as storage temperature. A shortened shelf-life is most often due to inadequacies in cleaning/ sanitizing practice that are likely to result in recontamination after the pasteurization process with organisms that grow under refrigeration and are capable of spoiling milk.

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Effects of Extrusion on Resistant Starch Content and Structure of Low and High Amylose Starches

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The enzyme resistance of regular maizes starch and two high-amylose maize starches was studied before and after extrusion. The starches, raw and extrudates, were analysed for resistant starch (RS) content, crystallinity by X-ray diffraction (XRD) and molecular order by FTIR. The high amylose starches, Gelose 50 and Gelose 80, had relatively high amount of resistant starch of about 42 and 45%, respectively. The amount of RS in regular maize starch was low at 0.5% which increased up to about 4 to 7 times after extrusion. However, there was a marked reduction in enzyme resistance of extruded high amylose starches, 1.4 to 4 times. Increasing feed moisture of starch during extrusion significantly increased the RS level in all starches. Storage of extruded starches at 4°C for 12 days increased RS content in regular maize starch but not in high amylose starches. XRD and infrared analysis showed crystalline and high molecular order in raw starches whereas extruded starches showed near amorphous structure. Interestingly there was still significant (>12%) enzyme-resistant starch in the extruded high amylose starches that were nearly amorphous.

Keywords: Maize starch, Amylose, Resistant starch, Extrusion, Crystallinity, Spectroscopy

Introduction

In plants, starch is synthesized in the form of water insoluble semi-crystalline granules with a complex architecture which is specific to each particular plant. Unlike other dietary polysaccharides, starches contain only á-glucosidic linkages and, are potentially digestible by the amylolytic enzymes secreted by the human digestive tract (Englyst & Hudson, 1996). Various studies, however, have shown that structural conformation and other factors can influence the rate and extent of starch hydrolysis,*in vitro* and *in vivo*, and subsequent absorption in humans and animals (Englyst *et al.*, 1992; Faisant *et al.*, 1993a & 1993b, 1995; Gidley *et al.*, 1995; Cairns *et al.*, 1996; Botham *et al.*, 1997). The sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals has been termed as 'resistant starch (RS)' (Englyst & Cummings, 1990; Asp, 1992). RS plays important physiological roles and has the potential to improve human health and lower the risk of many diet related non-infectious diseases. The foods with significant RS level may also have a low glycemic index (GI) value and hence have possible protective effects against type II diabetes, obesity and heart diseases (Kendell *et al.*, 2004). RS fraction not digested in the small intestine reaches the colon where it is largely digested and metabolized by gut bacteria into short chain fatty acids (SCFA), lactic acid and gases (Topping & Clifton, 2001). The SCFA, particularly butyrate, have been implicated in promoting good colonic health and preventing the incidence of colo-rectal cancer (Jacobasch *et al.*, 1999; Champ, 2004).

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RS is classified into 4 major groups, namely RSI, RSII, RSIII, and RSIV (Topping *et al.*, 2003). RSI arises from physically-inaccessible starches within e.g. plant tissue structures, RSII is due to the condensed form and partial crystallinity of native (uncooked) starch granules, RSIII is derived from recrystallised (retrograded) starches, typically after food processing, and RSIV is from chemical modification of starches that inhibit amylase digestion. RS3 or retrograded starch is the form of resistant starch most often used as an ingredient in a range of foods and is therefore of both commercial and nutritional interest.

Many food processing methods reduce or eliminate RSI and RSII but have the potential to generate RSIII. The currently accepted mechanism for the resistance of RSIII to amylase digestion is that linear amylose segments align themselves after gelatinization into condensed structures based on double helices (amylose retrogradation) that render the á-1, 4 glucosidic linkages inaccessible to amylase. Generally speaking, RSIII has been found to contain short and linear chains of thermally stable á-1, 4 glucans of about 10-100 glucose chain lengths, significant double helix content and moderate crystallinity (mostly B-type) (Eerlingen *et al.*, 1993a & 1993b; Gidley *et al.*, 1995, Shamai *et al.*, 2004, Cairn *et al.*, 1996; Faisant *et al.*, 1993a, 1993b, 1995). The mechanisms of starch aggregration, retrogradation, double helix formation, crystallization, chain length/molecular weight distribution and various physicochemical factors that lead to enzyme resistance of retrograded starch have been studied extensively (Eerlingen *et al.*, 1993 a, 1993b, 1994; Gidley *et al.*, 1995; Sievert & Pomeranz, 1989; Cairns *et al.*, 1996; Faissant *et al.*, 1993a, 1993b, 1995; Ring *et al.*, 1988). Many studies have shown that high amylose starches and products made from them

have high RS contents measured *in vivo* which correlate with enzyme-resistant values determined *in vitro* (Akerberg *et al.*, 1998; Thompson, 2005; Leeman *et al.*, 2006).

Extrusion cooking is a common food processing method for foods such as breakfast cereals, noodles and pasta products. Extrusion of starch in the presence of sufficient water triggers a number of physico-chemical and functionality changes in starch granules, such as the loss of granular structure associated with melting of crystallites and underlying helices, and generating an amorphous structure. This structure may later acquire ordered helical or crystalline order (= retrogradation) and become resistant to digestion by human á-amylase. Various investigators have reported that retrogradation following extrusion cooking results in formation of enzyme resistant starch (Kim *et al.*, 2006; Faraj *et al.*, 2004, Parchure & Kulkarni, 1997; Augustiniano-Osornio *et al.*, 2005; Bello-Perez *et al.*, 2005 Vasanthan *et al.*, 2002; Vasantham & Bhatti, 1998; Unlu & Faller, 1998; Eerlingen *et al.*, 1993a, 1994). In these studies, the acquisition of double-helical or crystalline order was considered to be critical for the observed amylase resistance. The inability of a starch double helix to fit into the active site of á-amylase is a plausible logic for the ability of retrograded structures to avoid enzymatic hydrolysis. In this study, three maize starches with various amylose contents have been subjected to extrusion. These extrudates were characterised by X-ray diffraction, infrared spectroscopy and *in vitro* resistant starch analysis. The aim of the research was to study the effect of extrusion processing on enzyme resistance and enumerate the molecular structure of the starch containing various levels of amylose.

Materials and Methods

Materials

Two high-amylose maize starches, Gelose 50 and Gelose 80, and a regular maize starch were purchased from Penfords Australia, Lane Cove, Sydney, Australia. The amylose contents of Gelose 50, Gelose 80 and regular maize starch were reported to be approximately 50, 80 and 27%, respectively. The raw starches were analysed for moisture content before extrusion experiment and kept in a sealed container at room temperature.

Extrusion of starches

Extrusion was carried out in a Prism Eurolab™ co-rotating twin screw extruder (Thermo Prism, Sataffodshire , UK). The screw diameter was 16 mm and the extruder barrel was 640 mm long, giving a length to diameter (L/D) ratio of 40:1. The extruder was divided into 10 different zones, the first of which was dry feed zone and rests were electrically heated barrel zones and a die block (with 2 openings of 2 mm dia). The barrel temperature profile was set at 50, 75, 100, 120, 120, 110, 105, 100, 95, and 80°C (die block). The screw speed was set at 180 rpm. The die block pressure and the motor torque were range from 6-24 bar and 11-42%, respectively, depending on the feed rate, water feed rate, type of starch and relative humidity of the extruder room. Dry feed was fed through a single screw feeder (KX16 Powder Feeder, Brabender Technology, Duisburg, Germany). Water was injected through a port of 150 mm from the start of the barrel using a peristaltic pump (L/S 7523) with a Tygon Lab tubing. The dry feed rate (Auger speed) was set at 25, giving a rate of 9-10g/min. The water feed rate was set at different rates, 1.5 to 7 g/min, depending on the amount of water need to be added on starch. Motor torque, screw speed, powder feed rate, barrel temperatures and melt pressure were monitored with Prismdde software (Sysmac-SCS version 2.2, Omron Corporation, UK).

Sample collection and storage

The extruder was set at a given set of barrel temperatures, screw speed, auger speed and higher liquid feed rate for about 15 minutes. Once the system get stable, the liquid feed rate was set at the level that gives extrudate with a particular moisture content e.g., 30, 40, 50%. The extrusion was carried out for 15-20 min before samples being collected. The moisture content of extrudate was immediately measured by Infra red moisture meter (Sartorius Moisture Analyzer, NY). Extrudates were cut into approximately 12 cm, cooled to room temperature, packed in Cryovac® bag, and stored for 0 (fresh sample) and 12 days at 4°C. The extrudates were dried overnight at 50°C in a vacuum oven (Thermoline Australia) and ground into powder by a hammer mill (Glen Creston Ltd, Stanmore, UK), The milled samples were analysed for moisture content by AOAC method 927.05 (AOAC, 1990), packed into air-tight plastic container and stored at room temperature. The extrusion plan is summarized in Fig. 1.

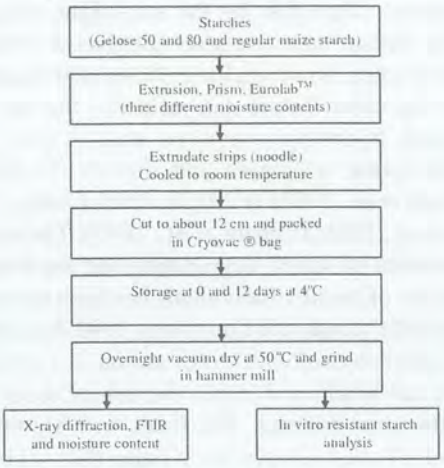

Fig 1: Flowchart for research plan.

In vitro Resistant starch analysis

The current method is an adaptation from Muir *et al.* (1995) which closely simulates starch digestion in humans. The ground extrudates were sieved and the particles retained between180-600 ìm sized sieves were used for RS analysis. The raw starches were in fine powder form and was analysed as is. About 500 mg of accurately weighed sample in a 125 mL conincal flask was mixed with artificial saliva [250U of á-amylase (Sigma A-3176) in carbonate buffer at pH 7.0]. After

15-20s, the mixture was incubated with acidified (0.02M HCl) pepsin (1 mg/mL; Sigma P-6887) at 37°C for 30 min. The solution was adjusted to pH 6.0 using 5 mL of 0.05 M NaOH and mixed with 25 mL of 0.2M sodium acetate buffer (pH 6.0). The buffered sample was incubated with 5 mL of pancreatin (2 mg; Sigma P-1750) and amyloglucosidase (28U, Sigma A-7420) enzyme mixture for 16 h at 37°C in a shaking water bath (Ratek Instruments, Boronia, Australia). The hydrolysed extract was transferred to 50 mL polypropylene centrifuge tubes and centrifuged at 2000g for 10 mins (Eppendorf, Model 5702R, Germany). The supernatant was discarded and the pallet was washed twice with 20 mL distilled deionized water, gently vortexing to break up the pallet. The pallet after centrifugation was resuspended in about 6 mL water and homogenized 3 times for 10 seconds in Ultra Turrax TP18/10 with an S25N-8G dispersing attachment (Janke & Kunkel IKA Labortechnik, Germany). The attachment was washed with water into the tube, to about 12 mL final volume. The homogenized mixture was carefully transferred to a 25 mL volumetric flask, mixed and poured back into the drained polypropylene tube. The solution was agitated for uniform suspension and 5 mL of extract was transferred to glass test tube (20x125 mm). It was frozen overnight at -20°C and freeze dried.

The extract contained mainly resistant starch fraction and now analysed for total starch content by enzymatic hydrolysis. The freeze dried samples and a reagent blank were wetted with 0.4 mL of 80% ethanol and vigorously vortexed to get a uniformly damp sample. Immediately, 2 mL of dimethyl suphoxide (DMSO) was added, vortexed and placed in a boiling water bath for 5 min. While tubes were in water bath, 3 mL of á-amylase (Megazyme E-BLAAM) in MOPS buffer (0.05 M, pH 7.0 with 0.5 mM CaCl2, 0.02% sodium azide, 1 mL enzyme in 30 mL buffer) was added to tube, vortexed and further incubated for 12 min with vortexing in between. The tube was placed in a 50°C water bath and 4 mL sodium acetate buffer (0.2M sodium acetate at pH 4.5, 0.02% sodium azide) was added. After a couple of minutes, 0.1 of amyloglucosidase (Megazyme E-AMGDF) was added, mixed and incubated at 50°C for 30 mins with mixing at the interval of 10 mins. The sample was transferred to 25 mL volumetric flask, made the volume, and an aliquot of extract was centrifuged at 2000x for 10 min.

The extract was analysed for the glucose content. One important aspect of this analysis was instead of using pure glucose for constructing a calibration curve, starch (S-5296, Sigma) and cellulose mixture at 4 different concentrations was used to simulate the matrix of starch rich foods. Starch/cellulose mixtures were hydrolysed and extracted along side test samples. The starch contents of these mixtures were measured before hand with glucose as standard using Enzyme Glucose Reagent (TR-15104, Thermoelectron, Victoria, Australia). A 50 iL of sample aliquot was transferred to a test tube and mixed with 1 mL Enzyme Glucose Reagent and incubated for 30 min at room temperature in the subdued light. The absorbance of tubes was read at 505 nm. The amounts of glucose present in hydrolysed starch/cellulose were first established using the regression equation from the calibration curve. Later a calibration curve from four different starch/mixtures was established and the glucose (and hence resistant starch) in samples were calculated based on regression equation. The procedure for RS analysis is also summarized in Fig. 2.

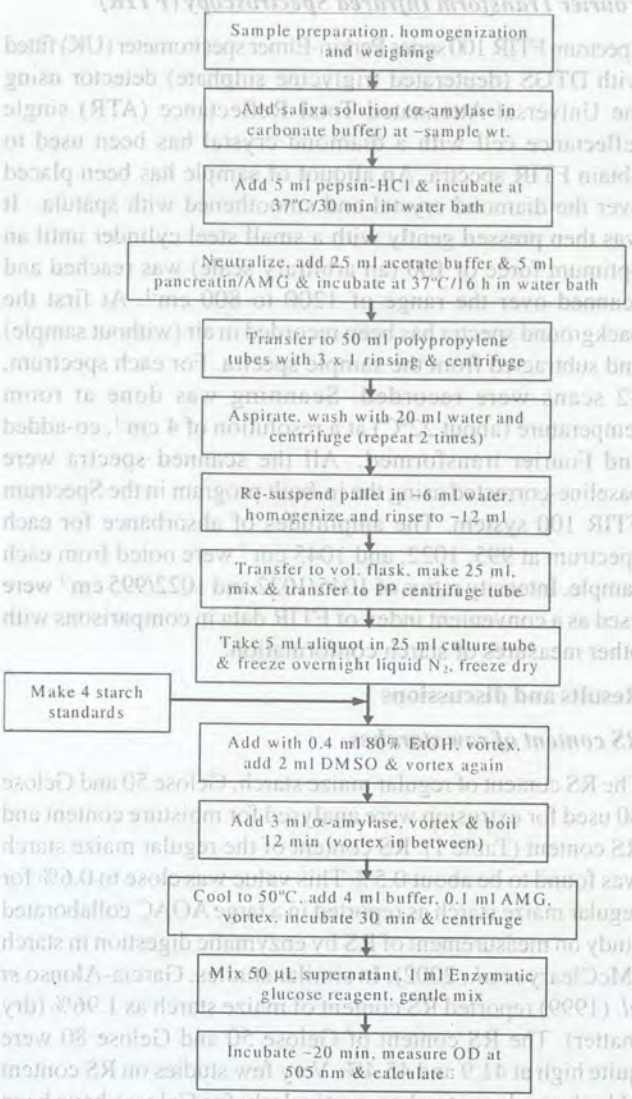

Fig 2 : Method for *in vitro* resistant starch determination and extraction.

The assay used Kellogg's Corn flakes as a standard reference material with a particular particle size, kindly provided by Commonwealth Scientific and Industrial Research Organization (CSIRO) Health and Nutrition (CHN), Adelaide, Australia. Only assays that gave acceptable RS values of 2.0-2.9% for reference material (Kellogg's corn flake) were accepted.

X-ray diffraction (XRD)

X-ray diffraction was carried out on a Bruker D8 Advance X-Ray Diffractometer equipped with a graphite monochromator,

Copper target, and scintillation counter (detector). The samples were examined over the angular range of 4 to 40 degrees with a step size of 0.1° and a count time of 2 seconds per steps. The total sampling time was 12 min. Crystallinity or amorphous nature of raw and processed starches were determined by using the EVA software.

Fourier Transform Infrared Spectroscopy (FTIR)

Spectrum FTIR 100 series Perkin-Elmer spectrometer (UK) fitted with DTGS (deuterated triglycine sulphate) detector using the Universal Attenuated Total Reflectance (ATR) single reflectance cell with a diamond crystal has been used to obtain FTIR spectra. An aliquot of sample has been placed over the diamond crystal and smoothened with spatula. It was then pressed gently with a small steel cylinder until an optimum force of 100 (an arbitrary scale) was reached and scanned over the range of 1200 to 800 cm^{-1}. At first the background spectra has been recorded in air (without sample) and subtracted from the sample spectra. For each spectrum, 32 scans were recorded. Scanning was done at room temperature (about 22°C) at a resolution of 4 cm^{-1}, co-added and Fourier transformed. All the scanned spectra were baseline-corrected using the in-built program in the Spectrum FTIR 100 system. The amplitudes of absorbance for each spectrum at 995, 1022, and 1045 cm^{-1} were noted from each sample. Intensity ratios of 1045/1022 and 1022/995 cm^{-1} were used as a convenient index of FTIR data in comparisons with other measures of starch conformation.

Results and discussions

RS content of raw starches

The RS content of regular maize starch, Gelose 50 and Gelose 80 used for extrusion were analysed for moisture content and RS content (Table 1). RS content of the regular maize starch was found to be about 0.5% This value was close to 0.6% for regular maize starch as reported in a large AOAC collaborated study on measurement of RS by enzymatic digestion in starch (McCleary *et al.*, 2002). In similar studies, Garcia-Alonso *et al.* (1999) reported RS content of maize starch as 1.96% (dry matter). The RS content of Gelose 50 and Gelose 80 were quite high at 41.9 and 45.4%. Very few studies on RS content of high amylose starches, particularly for Geloses have been reported in literature. Some of the commonly available high amylose starches such as I Ma HiHJJJJJHHHHJJKKFASDHi-Maize™ and Novelose® (both RS2) also reported to have high RS content of 42% (Champ, 2004). Recent analysis by this author (Htoon *et.al.*, 2007) found that Gelose 80 and Hylon VII (70% amylose) contained 45.7% and 60% RS (wet basis) which is comparable to current value. Comparison of RS content from a particular starch with values obtained in other studies, however, is difficult due to the different methodologies used (Goni *et al.*, 1996). Faisant *et al.* (1993b) reported three different RS values for three different methods (Englyst *et al.*, 1992) for retrograded and complexed starch.

Table 1: RS contents of raw regular maize starch and high amylose starches[1,2]

Samples	*RS (%)*	*Moisture (%)*
Regular maize starch	0.48 ± 0 (0.5)	11.9 ± 0.2
Gelose 50	41.9 ± 0.6 (47.8)	12.3 ± 0.2
Gelose 80	45.4 ± 2.4 (52.1)	13.2 ± 0.1

[1]All data are expressed as wet basis (values in parenthesis is in moisture free basis).
[2]RS content of standard reference material (cornflakes) was 2.19 ± 0.1%, within acceptable range

Various studies have shown that several factors can contribute towards the differences in RS quantities in foods such as: botanical origin; nature of starch (amylose and amylopectin content and their ratio); food processing (degree of starch gelatinization and retrogradation); starch morphology (particle size and cellular structure), kinds of starch granules or their crystalline structure (such as A, B and C or V) and presence of other components (lipids, protein, dietary fiber, antinutrients and organic acids etc (Goni *et al.*, 1996; McCleary *et al.*, 2002; Rosin *et al.*, 2002).

Effect of extrusion on RS content

The RS content of freshly extruded (also dried and ground) low and high amylose starches and their moisture content is given in Table 2. It shows the extrusion cooking of low amylose starch increased RS content by 4 to 6 times, depending on the moisture content of the extrudate. However, in high amylose starches extrusion caused a significantly drop in the RS content. The drop in RS content was higher in Gelose 50 (3-4 times) as compared to Gelose 80 (1.4 to 2.5 times), depending on the moisture content. The crystal forming the structure of starch granules (RS2) are disrupted by high pressure heating (>120°C and >10 Bar) in the presence of water which would be expected to increase accessibility of amylases to starch polymers. Upon cooling, hydrated amylose (and amylopectin) chains, under ideal conditions, would undergo a process of molecular re-association into double helices, stabilised by hydrogen bonds, and may consequently acquire resistance to enzymic digestion. Therefore, extruded products were expected to have higher RS content. It could be true that shearing action of the extruder screw in presence of high temperature may have caused degradation of longer amylose chains into small molecular fragments (less than 26 degree of polymerization) that could not be incorporated into a crystalline structure (Gidley *et al.*,1995). The fragmented starches may have remained more or less in amorphous condition. Another explanation could be that there was no new compensating or additional crystallites may have formed

Table 2 : RS contents of extruded regular maize and high amylose starches[1,2,3]

Samples	*RS (%)*	*Moisture (%)*
Regular Maize Starch (RMS)		
RMS, S1, 0 Day.	1.8 ± 0.02 (3.0)	40.0 ± 0.4
RMS, S 1, 12 Day.	2.2 ± 0.2 (3.7)	40.0 ± 0.4
RMS, S 2, 0 Day.	1.3 ± 0.0 (1.8)	29.0 ± 0.2
RMS, S 2, 12 Day.	1.6 ± 0.1 (2.2)	29.0 ± 0.2
Gelose 50		
Gelose 50, S 1, 0 Day	8.1 ± 0.3 (11.7)	30.7 ± 0.4
Gelose 50, S 1, 12 Day	8.3 ± 0.9 (12.0)	30.7 ± 0.4
Gelose 50, S 2, 0 Day	11.0 ± 0.1 (16.9)	35.1 ± 0.1
Gelose 50, S 2, 12 Day	11.2 ± 0.2 (17.2)	35.1 ± 0.1
Gelose 50, S 3, 0 Day	8.7 ± 6.1 (11.6)	24.8 ± 0
Gelose 50, S 3, 12 Day	8.6 ± .4 (11.4)	24.8 ± 0
Gelose 80		
Gelose 80, S 1, 0 Day	22.6 ± 0.3 (35.5)	36.3 ± 0.0
Gelose 80, S 1, 12 Day	22.6 ± 1.0 (35.5)	36.3 ± 0.0
Gelose 80, S 2, 0 Day	16.3± 0.1 (20.6)	20.8 ± 0.4
Gelose 80, S 2, 12 Day	16.5 ± 0.5 (20.9)	20.8 ± 0.4
Gelose 80, S 3, 0 Day	17.6 ± 0.0 (25.4)	30.4 ± 0.2
Gelose 80, S 3, 12 Day	18.5 ± 0.0 (26.6)	30.4 ± 0.2

[1]All data are expressed as wet basis (values in parenthesis is in moisture free basis).
[2]RS content of standard reference material (cornflakes) was 2.19 ± 0.1%, within acceptable range
[3]S means sample run.

between amylose chains in high amylose starches as extrusion temperature (~120°C) is probably below the threshold of starch fragmentation. Besides, the rapid drying that occurs in extrusion would not have resulted in extensive retrogradation. Any of these factors could be responsible for lower RS values in extruded starch. Faraj *et al.* (2004) also postulated similar theory for the decrease of RS content of barley flours during extrusion cooking. With regular maize starch, similar extrusion temperature in presence of water may have caused sufficient starch fragmentation of amylose (and some linear chains from amylopectin) leading to re-crystallization (and hence RS formation). Interestingly some studies have shown that extrudates of high amylose starches favour formation of RS more than extrusion of low amylose flours (Vasanthan *et al.*, 2002; Unlu & Faller, 1998).

There have been a number studies on effect extrusion on RS content of starches and starch rich foods. For example, our previous study showed that extrusion of high amylose starches such as Gelose 80 and Hylon VII significantly reduces the RS value by 3 to 4 times. The magnitude of RS reduction also depended on degree of extrusion, mild or extreme. Faraj *et al.* (2004) reported that extrusion followed by drying and grinding of barley flours decreases RS content,

especially at low moisture/very low or high temperature. Other extrusion studies also showed lower or similar RS values for e.g., barley flour (Ostergard *et al.*, 1989), rice and amaranth starch (Parchure & Kulkar, 1997) and wheat flour (Siljerstrom *et al.*, 1986).

Other investigators, however, have reported a higher yield of RS (up to 38%) when high amylose corn starch was extruded under high moisture conditions (Sievert & Pomeranz, 1989; Unlu & Faller, 1998). Kim *et al.* (2006) reported as high as 11 times increase in RS content of wheat pastry flour when extruded at higher feed moisture and stored at 4°C for 7 to 14 days.

Effect of feed moisture on RS content of extrudates

In this study, the individual starches were extruded at different feed moisture levels to study the effect of moisture on RS content of the extrudates and the results are shown in Table 2 and Fig. 3. The water feed rate during extrusion was set to achieve 30, 45 and 60% moisture in the extrudate. But it could not be achieved as the solid feeder was not feeding starch at constant rate. There was frequent feed build up in sample feeder. The moisture contents of freshly extruded samples (between 0 days and between 12 days) showed the extraction of starches at higher moisture content increased the RS content starch. The percentage increase in RS content of starches with increased moisture content (e.g. S1, S2 and S3) were almost same. For example, increasing moisture from 29 to 40% for RMS, both sample 1 and sample 2, gave 138% increase in RS content. In Gelose 50, it was noticed that slight increase in moisture did not increase the RS content e.g. 24.8 to 30.7 but increasing to 35.1% significantly increased the RS level. In Gelose 80 increase in RS content (16.5 to 22.5%) was more gradual with increasing feed moisture (21 to 36%). Kim *et al.* (2006) extruded pastry wheat flour at 20, 40 and 60% feed moisture at barrel temperatures similar to current study and screw speeds 150, 200 and 250 rpm. They reported an increase of 136% RS when feed moisture increased from 20 to 40% but about 500% increased when it increased from 20 to 60% moisture. This confirmed the previous studies that showed moisture acts as a plasticizer for retrogradation or starch, particularly in the range of 30-60% (Kim *et al.*, 2006)

Effect of storage on RS content of extrudates

The change in RS content of regular maize starch and high amylose starches when stored at 4°C for 12 day is given in Table 2. It shows there was about 1.25 times increase in RS content of regular maize starch during storage. In comparison, high amylose starches did not show any major change in RS content. Kim *et al.* (2006) showed about 2.5 times increase in RS content of pastry wheat flour during for 7 day storage at 4°C. Huth *et al.* (2000) reported up to 6% RS formation during extrusion of barley flour followed by storage at 4°C or -18°C for 3-7 days. However, no further increase of RS was observed when storage continued to 14 days. It seemed that the gelatinized and fragmented low amylose starches undergo molecular re-association (retrogradation) for first few days of

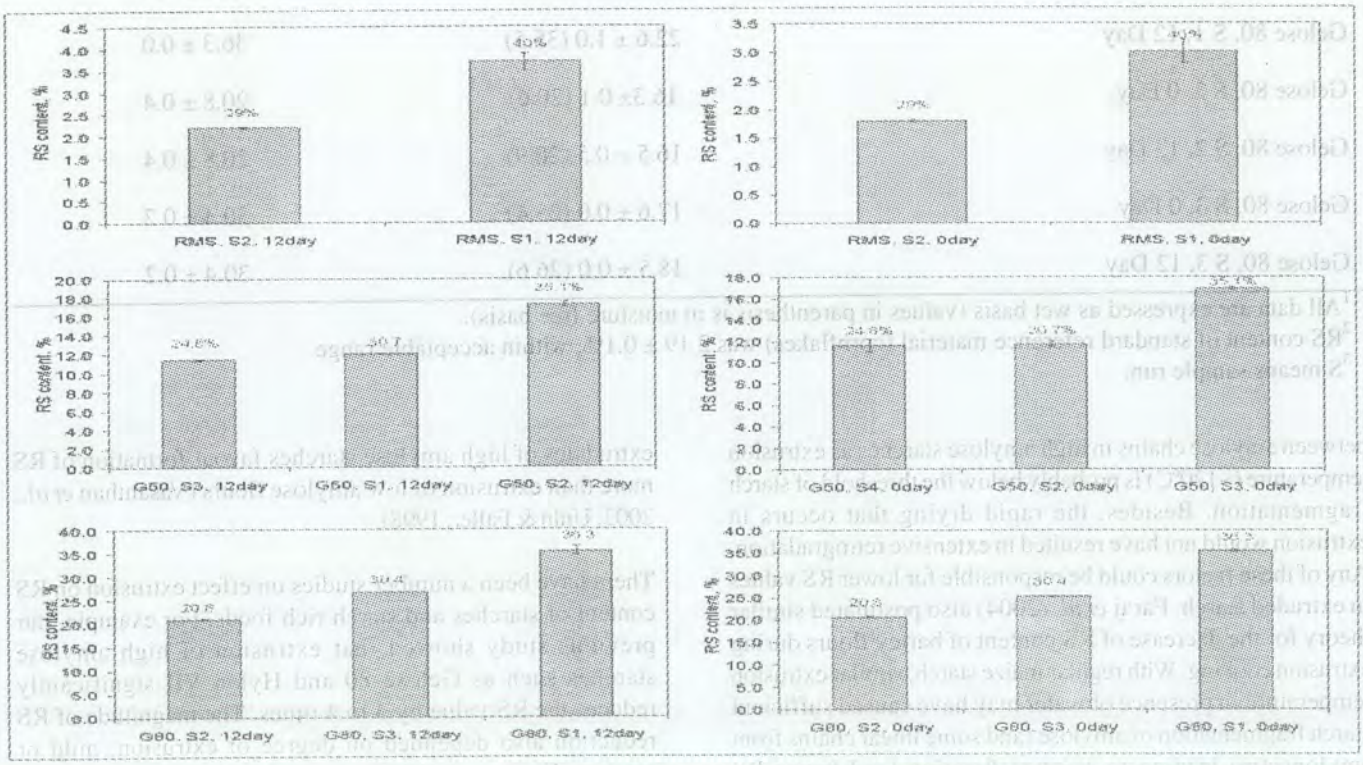

Fig 3 : RS (%dry basis) content of regular maize starch (RMS), Gelose 50 (G50) and Gelose 80 (G80) of extruded at various feed moisture and stored at 0 and 12 days at 4°C. Values at the top of the histograms are average moisture contents.

storage however, no such phenomenon may have occurred with high amylose starches.

The amount of RS in most of the commonly processed and grain based food products is relatively low, less than 0-3% (Sambucetti & Zuleta, 1996; Gelroth & Ranhotra, 2000; Goni *et al.*, 1997). There could have been some increment in RS content during extrusion but the overall RS content is still quite low and may not have significant impact on nutrition. High amylose starches in their raw states (RS2) contained high level of RS. Our study further confirmed that extrusion of these high amylose starches such as Gelose 50, Gelose 80, Hylon VII did not improve the RS content of their raw counterpart, even by using various extrusion conditions and or manipulating feed moisture (Htoon *et al.*, 2007).

X-ray diffraction (XRD)

The spectra of X-ray diffraction of raw, freeze dried, freshly extruded (0 day) and stored extrudates (4°C for 12 days) are shown in Fig. 4-6. The freeze dried forms of these starches were also scanned in X-ray diffraction to use as a reference for 100% amorphous sample. These figures suggested the semi-crystalline structure of raw starches being transformed into amorphous form due to disorganization of starch molecules during extrusion.

The raw regular maize starch showed a typical A-type crystalline structure with distinct peaks at angle 15° and 23° (Bello-Pérez *et al.*, 2005; Xie *et al.*, 2006) and an unresolved twin peak at 17° and 18° (Fig. 4). These distinctive peaks decreased significantly during extrusion as shown by spectra of extruded RMS (0 days). It indicated that the extruded RMS has lost much of its crystallinity but the structure was not totally amorphous as there were less intense, but distinct peaks at 15° and 23°. The peak heights of RMS stored at 12 days were more prominent than freshly extruded regular maize starch. The spectra of extruded regular maize starches were closer to A-type, as shown by the raw RMS.

The raw Gelose 50 and Gelose 80 showed a typical B-type structure of high amylose starches with distinct peaks at angle 17° and some other peaks at ~5°, ~20°, ~22.4° and 23.6° (Fi^gure 5 & 6), confirming similar finding by other researchers (Lopez-Rubio *et al.*, 2007; Xie *et al.*, 2006; van Soest *et al.*, 1995) The peak at ~5.5° is considered a fingerprint of this polymorph (Lopez-Rubio *et al.*, 2007). There was also some evidence for V-type peaks for example at 2θ ~20°. The freeze dried starch which is expected to be totally amorphous did show very small peaks. As in the case of regular maize starches, the signature peaks of B-type crystallinity in raw Gelose became less intense in extruded products. Contrary to our expectation, the extruded Gelose samples stored for 12 appeared to be less crystalline (more amorphous) than the freshly extruded ones. This study showed that the storage of extruded high amylose starch undergoes change in crystalline order. The product was more or less amorphous. However, there was still high

Fig 4 : X-ray diffraction of raw and processed regular

Fig 5 : X- ray diffraction of raw and processed Gelose 50

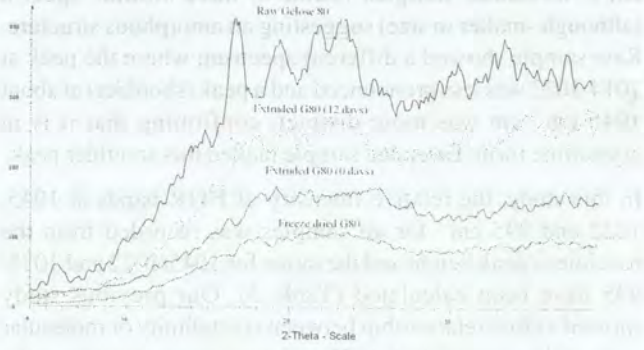

Fig 6 : X- ray diffraction of raw and processed Gelose 80

level of resistant starch (8.1–22.6%) in extruded high amylose starch with low level of crystallinity. Our previous study also showed a marked decrease in crystallinity of high amylose starches (Htoon *et al.*, 2007). It showed XRD %crystallinity for raw Gelose 80 and Hylon VII reduced from 17.6% and 17.4% to 2.1% and 2.4% for extruded Gelose 80 and Hylon VII, respectively. The high level of RS content in nearly 'amorphous' extrudates further confirms that crystalline order is not pre-requisite for significant enzyme resistance as they showed high RS content *in vitro*.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR-ATR spectroscopy has been used to further probe the changes in structure and physico-chemical properties of raw and processed starches. The representative FTIR spectra of raw and processed regular maize starch, Gelose 50 and Gelose 80 are shown in Fig. 7. Gelatinized (5% w/v starch boiled in water with stirring for 20 minutes) and freeze-dried regular maize starch was used as a reference for ~100% amorphous material. The spectral region of 800 to 1200 cm^{-1} was used in this study as previous studies have suggested that bands in this 'fingerprint' region reflect changes in polymer conformation and hydration of processed starches (Bello-Perez *et al.*, 2005; van Soest *et al.*, 1995; Goodfellow & Wilson, 1990). These studies also showed that band intensities at ca 1045, 1022 and 995 cm^{-1} are sensitive to changes in starch conformation as inferred from e.g. X-ray diffraction (Goodfellow & Wilson, 1990; van Soest *et al.*, 1995) or differential scanning calorimetry (Bello-Perez *et al.*, 2005). The bands at 1047 (or 1045) and 1022 cm^{-1} have been linked with order/crystallinity and amorphous regions in starch, respectively (Sevenou *et al.*, 2002; Bello-Perez *et al.*, 2005; van Soest *et al.*, 1995). Intensity ratios of 1045/1022 and 1022/995 cm^{-1} may therefore be useful as a convenient index of FTIR data in comparisons with other measures of starch conformation. Freeze dried samples showed a typical spectrum of an amorphous starch (Fig 7). Amorphous (freeze dried) structure seemed to have a pronounced peak at 1014-1022 cm-1. Extruded samples found to have similar spectra (although smaller in size) suggesting an amorphous structure. Raw sample showed a different spectrum where the peak at 1014-1022 was less pronounced and a peak (shoulder) at about 1045 cm^{-1} cm was more distinct, confirming that it is in crystalline form. Extruded sample lacked this shoulder peak.

In this study, the relative intensity of FTIR bands at 1045, 1022 and 995 cm^{-1} for all samples was recorded from the baseline to peak height and the ratios for 1045/1022 and 1022/995 have been calculated (Table 3). Our previous study showed a close relationship between crystallinity or molecular order at the ratio of 1045/1022 and amorphous structure at the ratio of 1022/995 (Htoon *et al.*, 2007). However, present study did not show any clear trend of higher crystallinity for 1045/1022 ratio and higher amorphousness for 1022/995 ratio. For example, RMS S1 (sample 1, regular maize starch) showed higher crystallinity and also higher amorphousness for raw

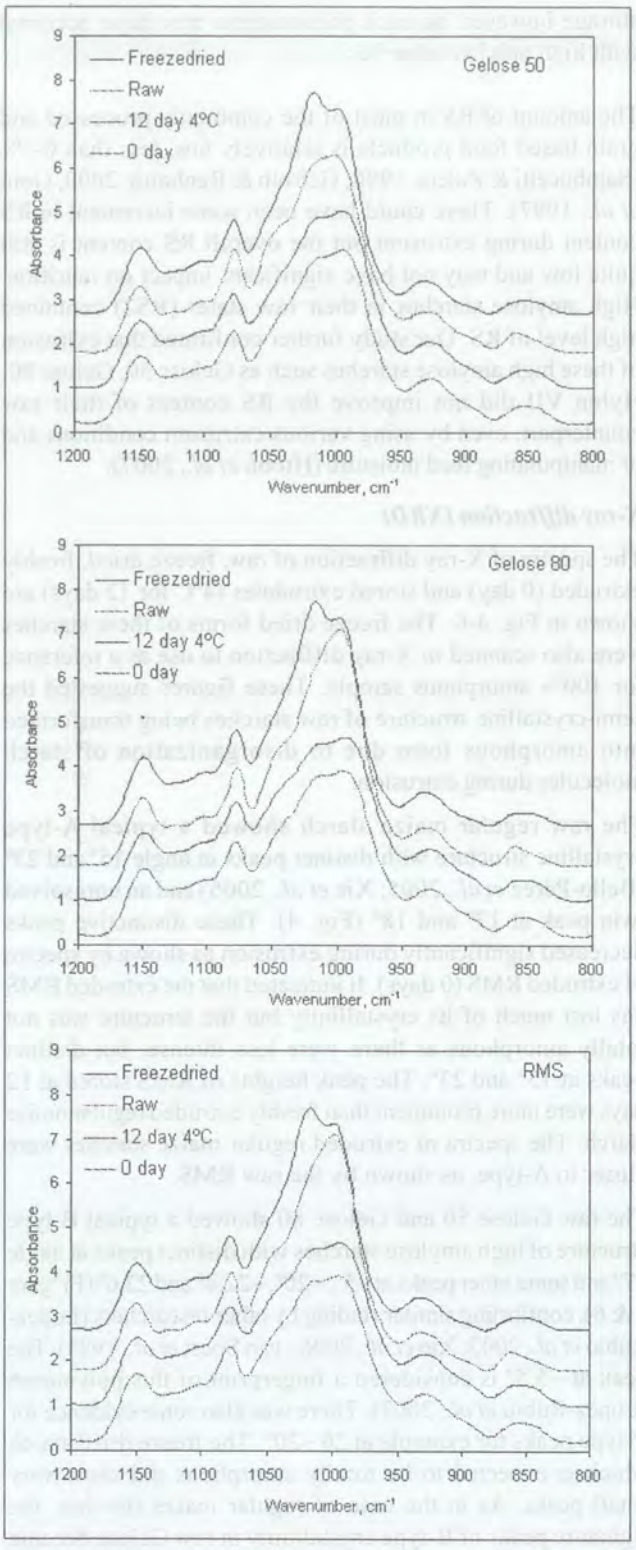

Fig 7 : FTIR spectra of representative Gelose 50, Gelose 80 and and regular maize starch at different storage conditions (spectra are arranged in the order as with sample notations).

starch compared to its extruded counterparts which cannot be true; sample with higher crystallinity should have lower amorphousness or *vice versa*.

Conclusions

This study showed the extrusion processing of low and high amylose starches alters the enzyme-resistance and structural confirmation. Extrusion slightly increased the resistant starch (RS) content of low amylose or regular maize starch. In high amylose starches (Gelose 50 and Gelose 80), a significant drop in RS was observed. Levels of RS were higher in extrudates that have been extruded at higher feed moisture. Storage of processed starches did not affect the RS content. The crystallinity and molecular order of extruded starches were low and close to the amorphous starch. Extruded high amylose starches were near amorphous but still had reasonably high RS content (>12%), suggesting molecular or crystalline order is not a pre-requisite for significant enzyme resistance.

Table 3 Comparison of peak heights of FTIR-ATR of raw and processed starches

Samples	*Peak ratios*	*Raw sample*	*0 day*	*12 days*
Regular Maize Starch (RMS)				
RMS, S1	1045/1022	0.723	0.693	0.711
RMS, S1	1022/995	0.717	0.697	0.714
RMS, S2	1045/1022	0.723	0.759	0.765
RMS, S2	1022/995	0.717	0.762	0.767
Gelose 50				
Gelose 50, S1	1045/1022	0.697	0.675	0.675
Gelose 50, S1	1022/995	0.932	0.883	0.894
Gelose 50, S2	1045/1022	0.697	0.673	0.700
Gelose 50, S2	1022/995	0.932	0.899	0.886
Gelose 50, S3	1045/1022	0.697	0.692	0.689
Gelose 50, S3	1022/995	0.932	0.901	0.879
Gelose 80				
Gelose 80, S1	1045/1022	0.710	0.707	0.644
Gelose 80, S1	1022/995	0.911	0.899	0.773
Gelose 80, S2	1045/1022	0.644	0.707	0.746
Gelose 80, S2	1022/995	0.773	0.909	0.908
Gelose 80, S3	1045/1022	0.644	0.694	0.713
Gelose 80, S3	1022/995	0.773	0.892	0.915

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Thermally Induced Gelation and Functional Properties of Porcine Leg Myofibrillar

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The effect of protein concentration and pH on protein solubility, gel strength, cooking loss and heat-induced gelation properties of porcine leg myofibrils was investigated. Myofibrils suspended in 0.6 N NaCl, pH 6.0 showed increasing gel strength and strongest gelling properties with increasing protein concentration. However, increasing protein concentration had a negative effect on cooking loss and protein solubility. In addition, leg myofibrils showed increasing protein solubility with storage at 4 °C. Results indicate protein solubility, gel strength, cooking loss and dynamic viscoelastic properties of porcine leg myofibrils are pH dependents. Highest gel strength was noted in pH 6.0, while highest protein solubility and lowest cooking loss in pH 7.0.

Keywords: Myofibrils, pH, Gelation, Protein solubility, Gel strength, Cooking loss

Introduction

Formation of protein gels in processed muscle foods is one of the most important functionalities, which influences the texture and sensory characteristics of finished products (Acton *et al.*, 1983) The myofibril, the smallest integral contractile unit of the muscle consists of highly organized complex of contractile proteins, i.e., myosin and actin, as well as regulatory and structural components (Porzio & Pearson, 1977) The isoforms and polymorphism of myosin and other myofibrillar proteins apparently are involved in producing the different functionalities in processed muscle foods (Morita *et al.*, 1987) Generally, myosin alone forms excellent gels. Actin has a synergistic or antagonistic effect on myosin gelation, depending upuon the myosin/actin ratio in the gelling system. Texture (Acton *et al.*, 1983; Macfarlane, 1977; Siegel & Schmidt, 1979; Asghar *et al.*, 1985) moisture retention and tenderness of processed muscle foods are influenced by the functionality of myofibrillar protein. Most investigations have indicated that myofibrillar proteins play a significant role in causing product variations among different muscle types (Morita *et al.*, 1987; Xiong & Brekke, 1991; Xiong, 1992) species (Lan *et al.*, 1995 a & 1995b) and pH Barbut and Mittal, 1993; Daum-Thunberg *et al.*, 1992).

Reported studies have identified large variations in functional properties of myofibrillar proteins associated with muscle fiber types. Myofibrillar proteins from fast- and slow-twitch fibers exhibit different biochemical and rheological characteristics and form gels with distinctly different viscoelastic properties and microstructures (Xiong, 1994) The significance of the differences in muscle fiber types in meat processing has been neglected in the past. However, recent studies suggest that the disparity in functional properties among various muscle or fiber types can be attributed mainly to myofibrillar proteins that are soluble in salt solution. Under similar processing conditions, myofibrillar protein derived from white muscle forms gels that exhibit quite different viscoelastic and rheological characteristics when compared with gels prepared from red muscle myofibrillar protein (Foegeding, 1987; Fretheim *et al.*, 1986; Robe & Xiong, 1993, 1994). Therefore, the objective of the study was to investigate the functional and rheological properties of porcine leg myofibrillar during storage and different pH conditions.

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Materials and methods

Materials

Post rigor pork leg meat (semimembranosus muscle) was purchased from a local market 24-36 h (pH 5.9 ± 0.15) post mortem. Two myofibrillar samples (replicates) were prepared on different days using the combined leg muscles from two animals in each preparation. All other chemicals were of analytical grade (Sinopharm Chemical Reagent Co Ltd, Shanghai, China).

Methods

Preparation of Myofibrillar

Myofibrills were prepared essentially according to Eisele & Brekke (1981) and all preparation steps were carried out at 4 °C. The de-bonned pork leg muscle was trimmed of heavy connective tissue & fat and ground through a 4.5 mm orifice plate in a food grinder (Model JCW 6 grinder, Shanghai Instrument Co., China). The ground muscle was blended in a waring blender (Model DS-1, Shanghai Instrument Co., Shanghai, China) in 4 volume of isolation buffer (0.1 N NaCl, 0.05 M sodium phosphate, pH 7.4, 5 mM EDTA and 1 mM NaN_3) for 90 sec at maximum speed. The suspension was stirred at 60 rpm with propeller for 30 min followed by centrifugation (model GL 20B, Shanghai Anting Science

Instrument Co., China) at 10000*g for 15 min. The resulting pellet was re-suspended in 4 volume (based on initial muscle weight) of the same buffer in the waring blender for 30 sec at maximum speed, then stirred with propeller for three 1 h intervals. Each stirring period was followed by a repeated centrifugation at 200*g for 15 min. The myofibrillar pellet was washed three times with 4 vol of 0.1 N NaCl solution containing 1 mM NaN_3 by homogenization for 30sec in the waring blender and centrifuged. The resulting myofibril pellet was re-suspended in 8 vol washing solution (0.1 N NaCl) and adjusted to pH 6.0 or specific pH (5.5 or 6.5 or7.0 or 7.5 for pH studies) with stirring using 0.1N HCl/ 0.1 N NaOH prior to centrifugation. The purified myofibrils (pellets) were stored at 0 °C and used within 3days of isolation.

Preparation of myofibril for measurement of protein solubility/extractability and gelation

Purified myofibrils were suspended by gentle stirring in NaCl solutions (containing 1 mM NaN_3) buffered with 50 mM Phosphate buffer, pH 6.0 or at specific pH for pH studies (5.5, 6.5, 7.0 or 7.5 for 24 h) at 4 °C. The amount of NaCl and the volume of the buffer were pre-determined so as to attain suspensions containing 0.6 N NaCl and various amounts of protein. The well mixed suspension was stirred manually at about 180 rpm for 5 min with a glass rod, and subsequently stored at 4 °C for various periods (up to 40 h). The 5 min stirring was sufficient to disperse the myofibrils while minimizing foaming.

Protein solubility / Extractability

To determine protein extractability (Xiong and Brekke, 1989), 8 ml aliquots of the myofibril suspension containing 5 mg mL^{-1} protein were placed in centrifuge tubes at specific time period (0, 10, 20, 30 and 40 h, pH 6.0 storage at 4 °C) or at specific pH (5.5,6.5,7.0 and 7.5 for 24 h storage at 4°C) or specific protein concentration (20 to 100 mg/mL for 24 h storage at 4 °C, pH 6.0) and centrifuged at 5000*g for 15 min at 4 °C. The protein concentration of the supernant was determined by the biuret method (Gornall *et al.*, 1949).

$$\text{Protein solubility/ Extractability \%} = \frac{\text{Protein concentration of the supernant}}{\text{Protein concentration of the original suspension}} * 100$$

Gel formation and measurement of gel strength

Myofibril suspensions were de-aerated prior to gelation by centrifuging the suspensions at 1000 rpm for 1 min. Twenty milliliter aliquots of the suspensions (20, 40, 60, 80 and 100 mg mL^{-1} protein for 24 h at 4 °C, pH 6.0), were placed in 25 mL beaker, or 20 mg mL^{-1} protein with specific pH (5.5, 6.0, 6.5 and 7.0 for 24 h at 4°C), covered with aluminum foil and heated in a 70°C water bath for 20min. The heated samples were immediately chilled in ice where they were kept until gel strength measurement. Gel strength was tested using the procedure of Hickson *et al.* (1982). A 1.2 cm diameter flat ended plunger was used to compress the heat induced gels to penetrate 60% original height and crosshead speed of 100 mm min^{-1}. The work of penetration (gel strength) (g*cm) for each treatment was measured. Samples were placed on the base of the TA-XT2i stable micro systems texturometer (Viena Court, England), taking care that the cylindrical probe reached the sample at the centre. Six samples were analyzed for each treatment. The texture expert version 1.2 software was used to collect and process the data.

Cooking loss (CL)

After measurement of gel strength, gel samples were then used for the cooking loss evaluation as described by Samejima et al., (1969). Compressed gels were centrifuged at 5000*g for 15 min. Cooking loss of the gels was calculated as

$$\text{Cooking loss \%} = \frac{\text{Slurry weight before heating - Gel weight after centrifugation}}{\text{Slurry weight before heating}} * 100$$

Dynamic rheological measurement

Myofibrils were suspended in 50mM sodium phosphate buffers with pH 6.5, 7.0 and 7.5 containing precalculated amounts of NaCl to produce a final NaCl concentration of 0.6 N and a protein concentration of 20 mg mL^{-1} or specific protein concentration (20, 40, 60, 80 and 100mg mL^{-1} at pH 6.0).

The dynamic oscillatory measurements of protein suspensions during gelation (thermal sol to gel transformation) were performed using an AR 1000 Rheometer (TA Instrument, West Sussex, UK) as described by Xiong (1993). Samples were loaded in the 1 mm gap between two parallel plates (upper plate diameter, 4.0 cm). The sample perimeter was covered with a thin layer of silicon oil to prevent dehydration and the plates were insulated in a foam-type shell to minimize heat dissipation. After equilibration at the initial temperature (20 °C) for 5min, samples was heated from 20 to 80 °C at a scan rate of 1°C min^{-1} by circulating water (from a programmable water bath) around the lower sample plate. The water temperature was controlled by software supplied with the rheometer. During heating, sample was sheared at a fixed frequency of 100 mHz with a maximum strain of 0.02. The actual sample temperature was verified using a thermocouple connected to the surface of the lower plate. Data were collected every minute during shearing measurement. Rheological properties of the myofibrillar gelling system (sol and gel) were continuously monitored in terms of storage modulus (G', is the rigidity due to the elastic response of the material), loss modulus (G", is the rigidity due to viscous response of the material) and phase angle (´, measures the phase difference between the stress and strain under imposed sinusoidal condition).

Statistical analysis

Two replicated experiments were performed using freshly prepared protein samples. All measurements were performed at least in triplicate. One-way analysis of variance with an F test, and Duncan Multiple Range Test (DMRT), were used to compare means and to identify significant differences ($P<0.05$) among treatments.

Results and discussion

Protein solubility / Extractability

Protein solubility of the myofibril suspensions, after specific storage time, specific pH and specific protein concentrations are shown in Fig. 1.

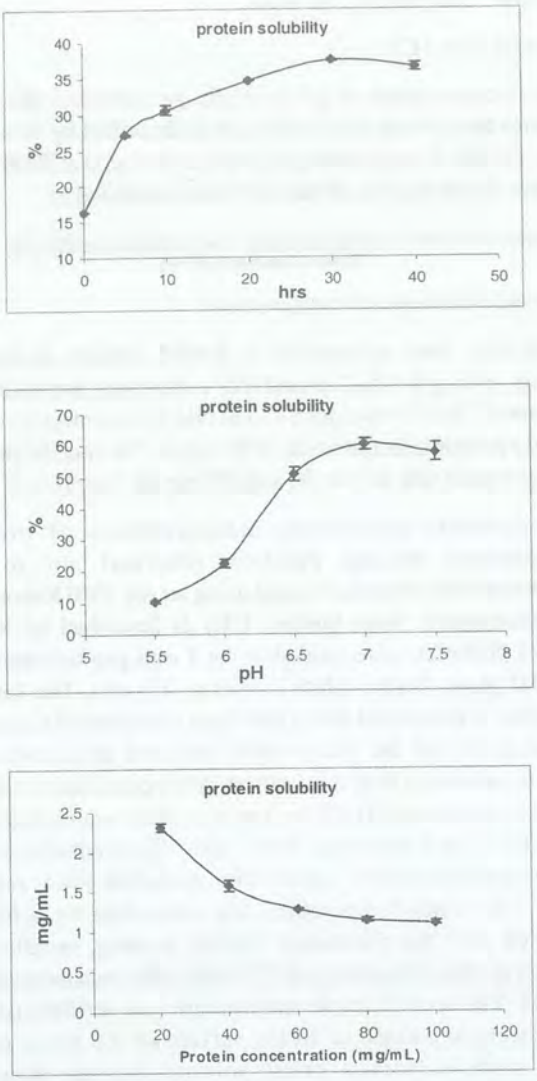


Fig. 1: Effect of protein concentration, storage time and pH on protein solubility of isolated porcine leg myofibrils.

There are no standard conditions for determining protein solubility by centrifugation (Xiong & Brekke, 1989). Leg myofibrils showed a rapid increase in protein solubility during the initial 10 h of storage. A further increase in protein solubility but rate is less than the initial values. However, highest solubility was observed in 30 h of storage and it was significantly ($P<0.05$) higher than the initial value. Because solubilization of proteins in the myofibrils was a continuous process, the salt extract should contain proteins solubilized at different times. The apparent increase in protein solubility of the myofibril suspensions during storage was only related to the dissociation of myofibrils; once the myofibrils were disassembled, the proteins could be readily solubilized. The integrity of the myofibrillar structure, and/or the formation of interfilament cross-linkages during rigor mortis, perhaps hindered the effect of salt to swell the myofibrils and subsequently to solubilize the proteins until sufficient storage time had elapsed. An increase in protein solubility during storage may have contributed to the increased water-holding capacity. It is clear that soluble proteins interact with water more favorably than if they are associated (myofibrils). In particular, the increasing water-holding capacity can be mainly attributed to the total amount of myosin solubilized, because myosin is largely responsible for water holding in meat systems (Nakayama & Sato, 1971). During postmortem storage, muscle protein is known to undergo biochemical changes. Dutson *et al.* (1974) observed substantial differences in morphological changes of porcine muscle up to 24 h at 2 °C. They showed white and red muscle fibrils were affected differentially, noting the disruption of Z-lines only in white fibrils, whereas red fibrils showed little or no change during this period. Presumably, alteration in Z-line would favor the release of certain myofibrillar proteins resulting in an increase in the amounts of proteins extracted from post rigor muscle. Lan *et al.* (1995a & 1995b) suggested that muscle with lower initial pH may undergo more protein denaturation which would reduce protein functionality and sensitivity to pH.

Leg myofibrils exhibited a pH dependent extraction profile. Protein extractability was least at pH 5.5, independent of muscle rigor state. This was probably because myofibrillar proteins collectively have an isoelectric point (p*I*) of pH 5.0-5.4. When the pH of a protein solution approaches the p*I*, there are decreasing net charges available for the protein molecules to interact with water and to repel each other, producing a reduction in protein solubility. Leg myofibrils showed a great dependence of protein extractability on pH between 6 and 6.5. This suggests an increase in electrostatic repulsion among protein molecules and increase in protein-solvent interaction as pH was increased from 6 to 6.5. At pH greater than 6.5, further change in protein extractability appeared to be minor. However, highest protein solubility was obtained at pH 7.0 and value was significantly ($P<0.05$) higher than the value at pH 5.5. A negative correlation was obtained between protein concentration and protein solubility. Our results also support with the findings of the Stanley *et al.* (1994). It is suggesting that swelling is associated with a higher level of protein solubilization and assumed that lower protein levels mean higher water levels and more swelling (Offer & Trinick,1983). Any treatment that leads to internal disruptions could promote swelling, perhaps by decreasing contact regions among protein subunits through repulsive forces.

Gel strength

Gel strength of the myofibrillar proteins was evaluated by the penetration force and the total work obtained from the force-

penetration curve. Since the overall patterns of changes in the total work resembled those of gel penetration force, both parameters may be used empirically to describe the gel strength. Fig. 2 show that the level of protein has an increasing effect on the binding ability of myofibrillar. These results may be attributed to a tighter matrix of interweaving fibers formed from the higher concentration of protein (Siegel and Schmidt, 1979). A maximum ($P<0.05$) in gel rigidity was established at pH 6.0 for myofibrillar, and gels formed at pH 7.0 were weaker than those prepared at pH 5.5, although the protein solubility followed the order of pH 7.0 > pH 6.5 > pH 6.0 > pH 5.5. Xiong (1994) reported that these results due to the inherent variations related to protein isoforms exist in myofibrillar protein and that protein solubility is not the sole factor affecting gel structure and gel strength.

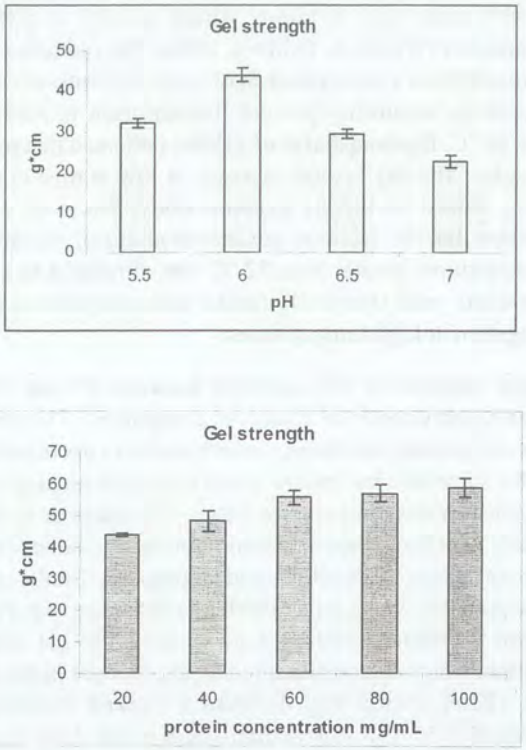


Fig. 2: Effect of protein concentration and pH on gel strength of myofibrils

Cooking loss (CL)

There was an effect of protein concentration and pH on water loss as shown in Fig. 3. Cooking losses decreased with increasing protein concentration and pH. The largest amount of water loss occurred at the lowest protein concentration, indicating that, not only was the available protein in the matrix not enough to make a strong gel, but also was not enough to effectively trap water. The consistent effect of pH on cooking loss indicates that water holding capacity of muscle protein was very dependent on pH. This suggests an increase in electrostatic repulsion among protein molecules and an increase in protein-solvent interactions as pH increased from 5.5 to 7.0. The highest cooking loss noted at pH 5.5, while lowest ($P<0.05$) value was obtained at pH 7.0. The increased protein-solvent interactions could increase the solubility of protein, thus improve water retention. Similar results were reported by Lan *et al.* (1995 a & 1995b).

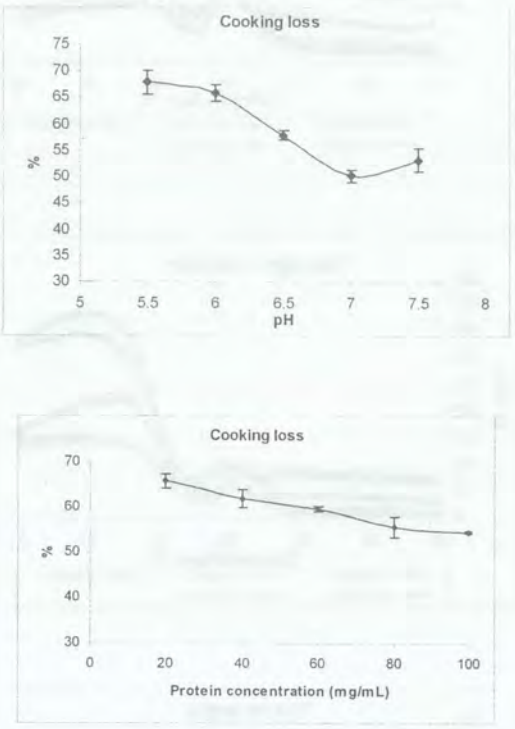
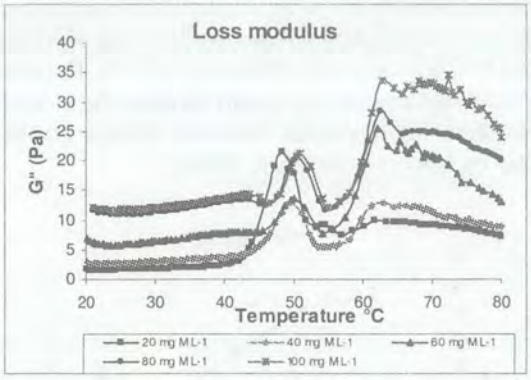


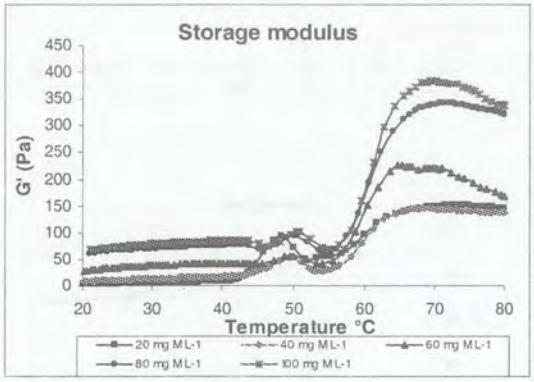
Fig. 3: Effect of protein concentration and pH on cooking loss of myofibrils isolated from porcine leg muscle tissues

Dynamic rheological measurement

(a) Effect of protein concentration on gelation

Dynamic viscoelastic measurements in all protein concentrations (20 to 100 mg/mL) showed similar patterns in gel viscoelasticity curves. A typical thermal scan is shown in Fig. 4. The average storage moduli (G′) of the samples were 145.6, 137.8, 169.4, 320.9 and 336.6 pa for the final gel (80 °C) for protein concentrations 20, 40, 60, 80 and 100 mg/mL, respectively. The G′ increased rapidly from 43 to 50 °C and hence, a greater probability for protein to form interpeptide crosslinks. Sharp & Offer (1992) reported that among the three light chains in myosin, only light chain 2 was involved in myosin gel networks. The myofibril suspension, which was virtually the mixture of salt soluble and insoluble protein, showed an intermediate increase in G′. After reaching a maximum at 50 °C, G′ sharply declined to a minimum at 55 °C and then steadily ascended showing a "shoulder" for myofibril samples. The multiple transitions in G′ indicate changes in the rate of forming elastic gel networks and reflect the different stages of structure unfolding and subsequent aggregation in myosin and actin. Myosin is comprised of





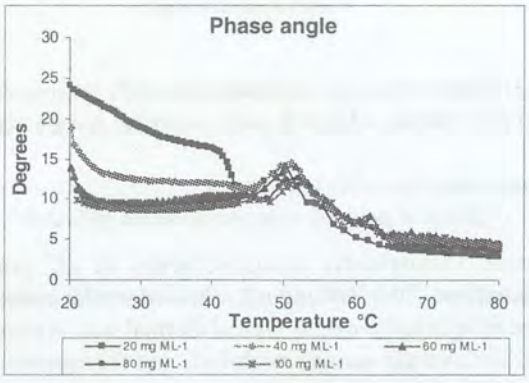


Fig. 4: Effect of protein concentration on thermal gelation of porcine leg myofibrillar suspension

different structural domains which unfold at different temperatures (Wright & Wilding, 1984). The reduction in G´ may result from a redistribution of intra- and intermolecular interactions, assuming protein denaturation is reversible below 55 °C. Egelandsdal *et al.* (1986) indicated that myosin molecular "fluidity" could increase at low temperatures of heating. Based on kinetic gelation study, Wu *et al.* (1991) suggested that the decrease in shear modulus of myosin gels at temperatures greater than 52 °C was attributed to kinetic constraints and thermodynamic unfavourableness for aggregation at high temperatures.

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A rapid increase in GÝ occurred between 40 and 50 °C, indicating prevalence of a viscous component. Therefore, a major conformational change, which caused a decrease in the mobility of protein but enhancement in peptide entanglement, took place in this temperature range. The sharp drop in GÝ after 50 °C reflects the reduction in protein volume fraction due to formation of loosely bound aggregates. Similar results were reported by Wang *et al.* (1990) for chicken protein. Protein gelation is characterized by a sol (viscous) to gel (elastic) transition, which can be measured by the changes in the phase angle´ (Ferry, 1980). Fig. 4 shows a marked increase in ´ between 45 to 50 °C for all myofibril reparations. After reaching a maximum at 50 °C, ´ dropped rapidly. This dynamic sequence would correspond to an initial protein structure unfolding followed by intermolecular interactions to produce a more elastic gel network. No apparent transitions were seen above 50 °C, suggesting that actin probably did not have a complex unfolding and aggregation process or had a minor contribution to protein gelation.

(b) Effect of pH on gelation

The effect of pH on thermal gelation of porcine leg myofibrillar suspension is given in Fig 5. During thermal gelation, myosin and salt-soluble myofibrillar protein exhibit complex changes in rheological characteristics depending on specific temperatures and pH (Xiong, 1993; Egelandsdal *et al.*, 1986; Wang *et al.*, 1990; Xiong & Blanchard, 1994).

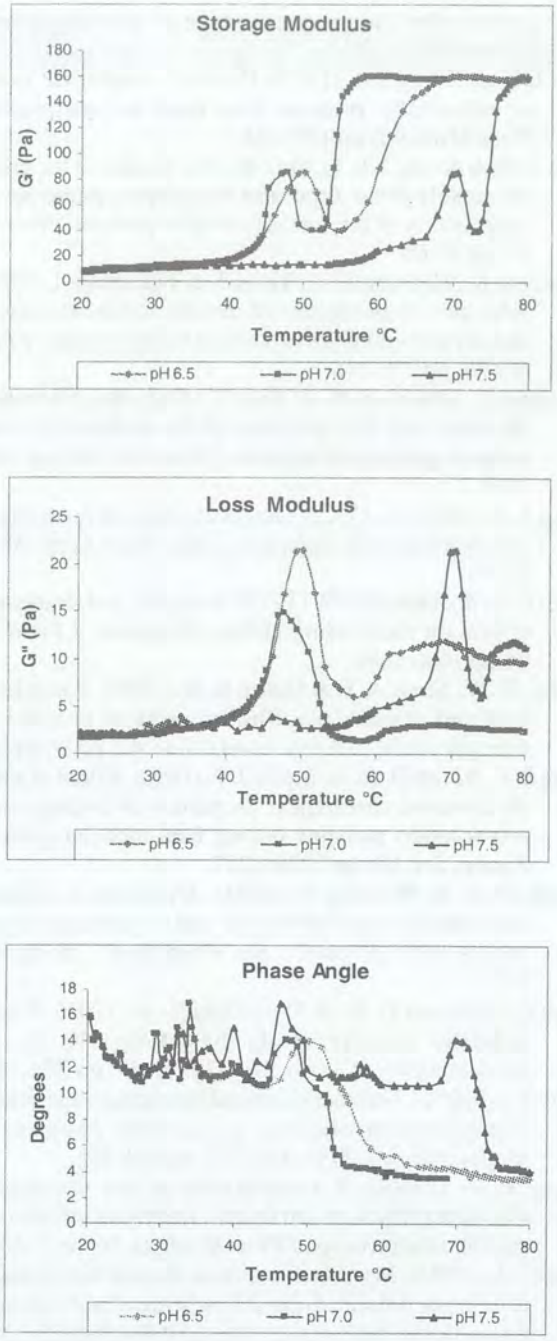

Fig. 5: Effect of pH on thermal gelation of porcine leg myofibrillar suspension

All samples began to form a gel, as indicated by an increasing storage modulus and decreasing phase angle, shortly after reaching a temperature of 42 °C in pH 6.5 & 7 and 68 °C in pH 7.5. The possibility of looser association between proteins at lower pH or decreased ability of hydrophobic interactions and disulfide bonds, both of which would allow for decreased ability for gelation, may be contributing factors to this observation. In beef loin protein, Egelandsdal *et al.* (1986) attribute the decrease and subsequent increase in the storage modulus to the denaturation of light meromyosin (LMM) followed by the formation of a gel of primarily denatured myosin molecules, suggesting increased gel strength and irreversible interactions. The mechanism by which myosin forms a gel is proposed to involve the aggregation of the heavy meromyosin (HMM), and the formation of a network amongst the LMM (Samejima *et al.*, 1981). Wright & Wilding (1984) reported that the transition temperatures of the sub fragments of myosin were affected by pH. They established, after examining pH 5.5, 6.0, 6.5 and 7.0, that LMM is more stable than HMM at pH 6.0, but becomes less stable with increasing pH. The differences in stability of HMM and LMM are a reasonable explanation for denaturation exceeding the rate of gel formation. In addition, model systems from a variety of protein sources have also reported similar depressions in storage modulus during the heating phase, when 1-3% protein is used (Kerry *et al.*, 1999a, 1999b & 1999c). Following the heating phase, all gels showed a phase angle of approximately 4°. The storage modulus remained constant during the holding phase, indicating completion of gelation at that temperature (80 °C).

Conclusions

Myofibrils prepared from porcine leg muscle contained a greater amount of salt-extractable protein and storage of myofibril suspensions has a positive effect on protein solubility. In addition, increased protein concentration had a positive effect on gelation properties and gel strength, while negative effect on cooking loss of the myofibril gel. Based on our results, it is clear that pH had a significant effect on porcine leg myofibrillar protein extractability, strength, cooking loss and gelation. Therefore, pH of the meat product should be carefully controlled to optimize the product functionality during ham processing when leg meat is used.

Acknowledgements

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Insoluble and Soluble Dietary Fiber Content in Vegetables Cooked by Different Methods

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Dietary fibre is an essential non nutrient with established physiological benefit. Vegetables are an important source of dietary fibre providing both soluble and insoluble fractions. The present study was undertaken to analyse the soluble and insoluble dietary fibre fractions in raw and cooked vegetables. Fifteen vegetables selected for the study were - Amaranth (Amaranthus gangeticus), Kilkeerai (Amaranthus tricolour), Shepu (Peucedanum graveolens), Spinach (Spinach Oleoracea), Beans (Phaseolus vulgaris), Brinjal (Solanum melongena), Knol khol (Brassica oleracea), Capsicum (Capsicum annum), Ladies finger (Abel maschus esculents), Green plantain (Musa sapientum), Radish (Raphanus sativus), Beetroot (Beta vulgaris), Carrot (Daucus carota), Potato (Solanum tuberosum) and Yam (Typohonum trilobatum). All the vegetables were processed using three domestic cooking procedures viz., conventional cooking by open pan boiling, pressure cooking and cooking in microwave oven. Raw vegetables served as controls. Soluble (SDF) and insoluble dietary fibre (IDF) was analysed in all the samples by enzymatic/gravimetric assay. Results revealed that on dry weight basis the IDF content of greens ranged from 24.72 - 33.87 g% and SDF ranged from 5.27 - 6.98 g%. In other vegetable group the values were 11.73 – 37.31g% and 1.89 – 8.24 g% for IDF and SDF respectively. Roots and tubers had much lower content of IDF and SDF, 11.14 - 16.79 and 1.57 -3.33 g% respectively. On fresh weight basis the IDF content of vegetables ranged from 1.60 - 3.15 g% and SDF ranged from 0.12 - 0.94 g%. On cooking, in general, a decrease was seen in IDF content of vegetables whereas an increase was seen in SDF. However the extent of increase or decrease varied with the vegetable. There were no differences observed in dietary fibre of vegetables cooked by three different methods.

Keywords : Soluble Dietary fiber, Insoluble Dietary fiber, Vegetable, Cooking

Introduction

Dietary fiber is defined chemically as the sum of polysaccharides and lignin, which are not digested by the endogenous secretions of the human gastrointestinal tract. Various components that make up the fiber include, cellulose, hemicellulose, lignin, pectin, gums, mucilage and algal polysaccharides (Torre *et al.*, 1991). Plant foods, especially fruits and vegetables have been found to be good sources of dietary fiber. Composition and properties of plant fiber vary greatly depending on various factors like growth, location, storage, source, species and part of the plant (Mullin & Wolynetz, 1995; Svanberg & Nyman, 1997).

Dietary fiber is reported to have several physiological effects depending upon the physical and chemical nature of the individual fiber source. Fibers of fruits and vegetables are known to improve the bowel function and increase fecal bulk. Sources of noncellulosic polysaccharide that are soluble and viscous are found to be effective in reducing plasma cholesterol levels and glycemic responses to a meal (Schneeman, 1989). But increased consumption of dietary fiber may also have certain negative effect on the utilization of minerals (Joshi & Agte, 1995).

Insoluble dietary fiber comprises of cellulose, insoluble hemicellulose and non polysaccharide lignin. Soluble fiber comprises of soluble hemicellulose, pectins and gums (Pak *et al.*, 1989). Methods for the determination of dietary fiber are divided into four categories, namely, gravimetric, gravimetric-enzymatic, calorimetric and chromatographic. Gravimetric techniques were the earliest and included crude fiber, acid detergent fiber (ADF) and neutral detergent fiber (NDF). These methods grossly underestimate dietary fiber content and are being replaced by new and more accurate methods (Dreher, 1987). Comparison of four different gravimetric methods for the determination of fiber content of foods shows that the crude fiber method gives an inaccurate estimation of cellulose and lignin contents in foods and does not estimate noncellulose polysaccharides. The NDF primarily estimates the structural components of the cell wall – cellulose, hemicellulose and lignin. The soluble fiber sources, gums and pectins must be estimated by other methods. By itself, NDF underestimates the total fiber content of a food, but in conjunction with an analysis for soluble polysaccharides, it is useful. Lignin and cellulose components with small amounts of hemicellulose and pectin are detected by the acid detergent method (ADF). The difference between NDF and ADF provides a rough estimate of the hemicellulose content. The loss of soluble fibers associated with these methods however, leads to an underestimation of the total fiber content. Unlike the other analytical methods, all of the fiber fractions are included as part of a total fiber estimation by the enzymatic procedure. Results from this procedure are most likely a slight

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overestimation of fiber content because of potential contributions from starch or heated proteins (Schneeman, 1989).

Vegetables being high in water content, effectively dilute the dietary fiber content. With the exception of seed legumes the values for total dietary fiber in fresh vegetables are often less than 5%. In leafy vegetables collenchyma with nonlignified walls are a major element in maintaining the erect plant in contrast to the monocotyledonous grasses where lignified sclerenchyma are important structural elements. These cells in turn are the major contributors of dietary fiber. The noncellulosic polysaccharides of leafy vegetables are known to contain similar proportions of pentoses and uronic acid, whereas in root vegetables, uronic acid forms the major portion (Southgate, 1978).

The present investigation focused on analyzing the dietary fiber contents of raw and cooked vegetables by gravimetric – enzymatic method to get the true fiber values.

Methodology

A total of fifteen vegetables belonging to three categories namely 1. Green leafy vegetables group, viz., amaranth (*Amaranthus gangeticus*), kilkeerai (*Amaranthus tricolour*), shepu (*Peucedamum graveolens*) and spinach (*Spinach oloeracea*), 2. Other vegetables group, viz., beans (*Phaseolus vulgaris*), brinjal (*Solanum melongena*), knol khol (*Brassica oleracea*), capsicum (*Capsicum annum*), ladies finger (*Abel maschus esculents*) and green plantain (*Musa sapientum*), 3. Roots and tubers group, viz., radish (*Raphanus sativus*), beetroot (*Beta vulgaris*), carrot (*Daucus carota*), potato (*Solanum tuberosum*) and yam (*Typohonum trilobatum*) were selected for the study. The vegetables were purchased in one lot on the day of processing from the local market. The vegetables were sorted, washed, peeled/trimmed, cut and divided into four equal parts after thorough mixing. One part was retained as such i.e., raw, which served as a control and the other three parts were subjected to three different cooking methods namely,

a) Conventional, boiling : one part of cut vegetable + water (distilled), covered and cooked on gas (medium – flame) with occasional stirring till done. Doneness was tested by finger feel. Ladies finger were shallow fried in oil without adding any water.
b) Pressure cooking : one part of cut vegetable + water (distilled) cooked in a pressure cooker (15lbs) on gas (medium – flame). Ladies finger were not processed by this method.
c) Microwave cooking : one part of cut vegetable + water (distilled) cooked in a round, Borosil glassware with lid using only high power. The vegetables were stirred occasionally while cooking. Doneness was tested by finger feel. Ladies finger were processed using small amount of oil as in shallow frying with frequent stirring.

Water required and the time taken for cooking (that resulted in similar products) was standardized for each vegetable and for each cooking method individually before the actual cooking of the vegetables for the purpose of analysis was done.

Raw and cooked samples were spread uniformly as a thin layer on separate aluminum plates and dried in a electric oven at around 60°C for 16-22 h. The powdered samples were packed in polythene covers and stored in tight plastic container and kept under refrigeration until required for analysis.

Dietary fiber content was estimated by the enzymatic-gravimetric method of Asp *et al.* (1983) The digestible starch and protein was removed using Pepsin (Art-7187, Merck, Germany) and Diastase (49013, Sd Fine chemicals, India) and Termamyl (120KNU/g, AAN 4306, NOVO Co. Denmark). The water insoluble dietary fiber was filtered (residue) and filtrate containing soluble fiber is precipitated. Corrections were made for protein and mineral content of the residue. The data was analyzed using standard deviation and ANOVA.

Results and Discussion

The insoluble dietary fiber (IDF) contents of raw and processed vegetables are presented in Table 1. Green leafy vegetables had a range of 1.31 to 3.15 g/100g of IDF, which showed a decrease in three methods of cooking for amaranth, kilkeerai and shepu. The IDF in spinach was lowest i.e. 1.31g/100g but it showed an increase on cooking. On dry weight basis, such differences were not seen. The IDF ranged from 24.72 to 33.87g/100g in dried samples. On cooking the values were much closer and ranged from 21.89 to 29.18g/100g for conventionally cooked, 24.18 to 30 .74 for pressure cooked and 22.48 to 26.91 for microwave cooked samples.

In the other vegetable group, a wider range of IDF values in raw vegetables was seen. Capsicum and knol khol were in the range of 1.53 to 1.60, green plantain and brinjal, 2.17 to 2.72 and beans and ladies finger, 3.00 to 3.75g/100g. Of these, beans, brinjal and knol khol showed a reduction in samples cooked by all methods. Capsicum was lesser for conventionally cooked and microwave cooked samples but slightly higher for pressure cooked samples. Ladies finger was low for conventionally cooked (shallow fried in oil) sample but higher for microwave cooked sample. Green plantain on the contrary showed an increase in IDF content on cooking by all methods. Similar trend was also seen on dry weight basis with the exception of microwave cooked ladies finger sample.

Roots and tubers relatively had lesser IDF content with values ranging from 1.68 to 2.45g/100g. On cooking beetroot, potato and yam showed an increase in IDF content although the extent of increase varied depending upon the method of cooking. Carrot and radish exhibited a decrease. On dry weight

Table 1. Insoluble dietary fiber in raw and processed vegetables (g/100g fresh basis)

Vegetable	*Raw*	*Conventionally cooked*	*Pressure cooked*	*Microwaved*	*F Ratio*
Amaranth	3.15±0.14 (33.87)	2.86±0.29 (29.18)	2.72±0.39 (27.2)	2.94±0.18 (26.97)	0.957 ns
Kilkeerai	2.87±0.38 (31.89)	2.74±0.33 (28.54)	2.92±0.50 (30.74)	2.61±0.34 (26.91)	0.225 ns
Shepu	2.92±0.41 (30.42)	2.51±0.09 (23.90)	2.41±0.21 (26.19)	2.63±0.86 (22.48)	0.387 ns
Spinach	1.31±0.13 (24.72)	1.62±0.24 (21.89)	1.62±0.05 (24.18)	1.59±0.05 (22.71)	0.284 ns
Beans	3.00±0.57 (33.71)	2.34±0.15 (24.63)	2.58±0.35 (31.08)	2.39 ±0.24 (28.12)	1.387 ns
Brinjal	2.72±0.02 (37.31)	2.51±0.25 (33.92)	2.25±0.28 (35.16)	2.75±0.37 (32.35)	1.533 ns
Capsicum	1.53±0.06 (22.50)	1.40±0.04 (18.52)	1.64±0.36 (23.10)	1.47±0.56 (19.34)	0.592 ns
Knol Khol	1.60±0.26 (22.54)	1.44±0.39 (18.70)	1.27±0.24 (19.24)	1.14±0.34 (17.56)	0.368 ns
Ladies finger	3.75±0.09 (34.63)	3.51±0.07 (27.64)	-	4.28±0.75 (27.61)	1.626 ns
Plantain, green	2.17±0.02 (11.73)	2.84±0.86 (14.79)	2.85 ±1.42 (13.77)	2.84±0.92 (12.14)	0.247 ns
Beetroot	1.68±0.23 (17.68)	1.99±0.99 (17.77)	1.78±0.57 (20.94)	2.43±1.33 (21.70)	0.287 ns
Carrot	2.45±0.13 (18.56)	2.42±0.18 (17.66)	1.80±0.03 (14.17)	2.46±0.13 (17.83)	1.250 ns
Potato	1.85±0.39 (11.14)	2.56±0.95 (14.97)	1.99±0.39 (11.78)	2.42±0.15 (12.60)	0.724 ns
Radish	2.23±0.80 (41.29)	1.74±0.75 (29.00)	1.63±0.55 (33.27)	1.97±0.76 (30.31)	0.266 ns
Yam	2.26±0.13 (13.61)	2.63±0.48 (14.06)	2.39±0.11 (16.79)	2.72±0.05 (14.17)	1.386 ns

Values represent mean SD of duplicate samples of two individual batches. Figures in parenthesis represent values on dry weight basis.

basis similar trend was seen. However, none of the differences seen due to cooking methods were statistically significant for any vegetable.

The soluble dietary fiber (SDF) content of vegetables is presented in Table 2. In general, all the vegetables had much lesser content of soluble fiber in comparison with insoluble fiber. The SDF of greens ranged from 0.32 to 0.62 g/100g which showed an increase in all vegetables on cooking. The extent of increase varied depending on the type of vegetables. For eg. Amaranth showed an increase of 35, 51 and 57 % in conventional, pressure and microwave cooked samples respectively. These values were statistically significant in comparison with control. Kilkeerai, shepu and spinach showed a much lesser increase ranging from 2-30%.

Between different cooking methods, microwave cooked samples had a slightly higher increase in SDF content. Such differences were not apparent on dry weight basis. Some vegetables on cooking exhibited lower SDF content. These include conventionally cooked kilkeerai, shepu and spinach, pressure cooked kilkeerai and spinach and microwave cooked shepu and spinach.

Table 2. Soluble dietary fiber in raw and processed vegetable (g/100g fresh basis)

Vegetable	*Raw*	*Conventionally cooked*	*Pressure cooked*	*Microwaved*	*F ratio*
Amaranth	0.49 [a] ±0.02 (5.27)	0.66 [b] ±0.03 (6.73)	0.74 [b] ±0.04 (7.40)	0.77 [b] ±0.03 (7.06)	23.459 *
Kilkeerai	0.48 ±0.11 (5.33)	0.49 ±0.04 (5.10)	0.46 ±0.01 (4.84)	0.63 ±0.16 (6.49)	1.107 ns
Shepu	0.67 ±0.07 (6.98)	0.69 ±0.05 (6.57)	0.67 ±0.12 (7.28)	0.76 ±0.13 (6.49)	0.398 ns
Spinach	0.32 ±0.02 (6.04)	0.38 ±0.13 (5.14)	0.33 ±0.02 (4.93)	0.41 ±0.21 (5.86)	0.204 ns
Beans	0.55 ± 0.04 (6.18)	0.61 ±0.07 (6.42)	0.53 ± 0.15 (6.39)	0.60 ±0.03 (7.06)	0.457 ns
Brinjal	0.22± 0.13 (3.01)	0.38 ±0.12 (5.14)	0.45 ± 0.10 (7.03)	0.51 ±0.05 (6.00)	0.246 ns
Capsicum	0.60 ±0.25 (6.03)	0.62 ±0.13 (5.81)	0.53 ±0.09 (5.77)	0.63 ± 0.15 (5.79)	0.128 ns
Knol Khol	0.22 ±0.02 (3.10)	0.20 ±0.06 (2.60)	0.22 ±0.09 (3.33)	0.22 ±0.06 (2.68)	0.056 ns
Ladies finger	0.67[a] ±0.23 (8.24)	1.12 [b] ± 0.04 (7.40)	-	1.65 [b] ± 0.17 (9.03)	17.144*
Plantain, green	0.35 ±0.0 (1.89)	0.36 ±0.28 (2.39)	0.44 ±0.21 (2.13)	0.49 ±0.01 (2.09)	0.179 ns
Beetroot	0.38 ±0.05 (4.00)	0.40 ±0.01 (3.57)	0.46 ±0.06 (5.41)	0.47 ±0.06 (4.20)	1.478 ns
Carrot	0.94 ±0.12 (5.91)	1.16 ±0.22 m (6.14)	1.09 ±0.20 (6.54)	1.04 ±0.06 (6.09)	0.657 ns
Potato	0.26 ± 0.11 (1.57)	0.55 ± 0.14 (3.22)	0.53 ± 0.11 (3.14)	0.43 ± 0.09 (2.24)	2.699 ns
Radish	0.12 ±0.01 (2.22)	0.16 ±0.03 (2.67)	0.16 ±0.01 (3.27)	0.26 ±0.17 (3.00)	0.951 ns
Yam	0.35 ± 0.44 (2.11)	0.54 ± 0.01 (2.89)	0.54 ± 0.16 (3.33)	0.50 ± 0.07 (2.60)	2.232 ns

Values represent mean SD of duplicate samples of two individual batches. Value with different superscripts in a column (for a specific vegetable) are significantly different. Figures in parenthesis represent values on dry weight basis.

The SDF content of other vegetables were in the range of 0.22 to 0.67g/100g. Among conventionally cooked vegetables all showed an increase except knol khol, among pressure cooked vegetables beans and capsicum showed an increase. A wide variation was noticed in the extent of increase or decrease. On dry weight basis there were slight changes in the trend, showing an increase for all cooked samples of beans, brinjal and plantain and showing a decrease in capsicum. For knol khol and ladies finger it was high for pressure cooked and microwave samples respectively.

The SDF content of roots and tubers ranged from 0.12 to 0.94g/100g. Carrot had highest SDF among all vegetables analysed. On cooking a very consistent pattern of increase was observed in all vegetables of this group. Potato showed highest increase followed by radish, yam, beet root and carrot. The total dietary fiber content computed for insoluble and soluble dietary fiber (Table 3) shows a higher fiber content in amaranth, kilkeerai and shepu and in general, a decrease is seen on cooking in all samples. Ladies finger had highest amount of total dietary fiber. In spinach, beet root, green plantain, potato and yam the total dietary fiber content was

Table 3. Total dietary fiber in raw and processed vegetables* (g/100g fresh basis)

Vegetable	*Raw*	*Conventionally cooked*	*Pressure cooked*	*Microwaved*
Amaranth	3.64	3.52	3.46	3.71
Kilkeerai	3.35	3.23	3.38	3.24
Shepu	3.59	3.20	3.08	3.39
Spinach	1.63	2.00	1.95	2.00
Beans	3.55	2.95	3.11	2.99
Brinjal	2.94	2.89	2.70	3.26
Capsicum	2.13	2.02	2.17	2.1
Knol khol	1.82	1.64	1.49	1.36
Ladies finger	4.42	4.63	-	5.93
Plantain, green	2.52	3.20	3.29	3.33
Beet root	2.06	2.39	2.24	2.90
Carrot	3.39	3.58	2.89	3.50
Potato	2.11	3.11	2.52	2.85
Radish	2.35	1.9	1.79	2.23
Yam	2.61	3.17	2.93	3.22

*: Computed from insoluble and soluble dietary fiber.

lower but an increase was seen on cooking. Consumption of 300-500 g of vegetables in a diet as is generally advised will contribute 10 to 16 g of fiber depending upon the variety of vegetable consumed and can provide about 40-50% of daily requirement of fiber for an ideal diet.

The effect of cooking in general on the IDF and SDF of vegetables analysed as mean increase or decrease with reference to the respective raw controls is depicted in Fig. 1 and 2. As can be seen from the graph, cooking brought about a decrease in IDF content of most of the vegetables analysed. An increase however was seen in spinach, ladies finger, green plantain, beetroot, potato and yam. The soluble fiber was increased for all vegetables with the exception of capsicum and knol khol where it was almost similar to control.

Fig. 1. Effect of cooking on the insoluble fiber content of vegetables in relation to control

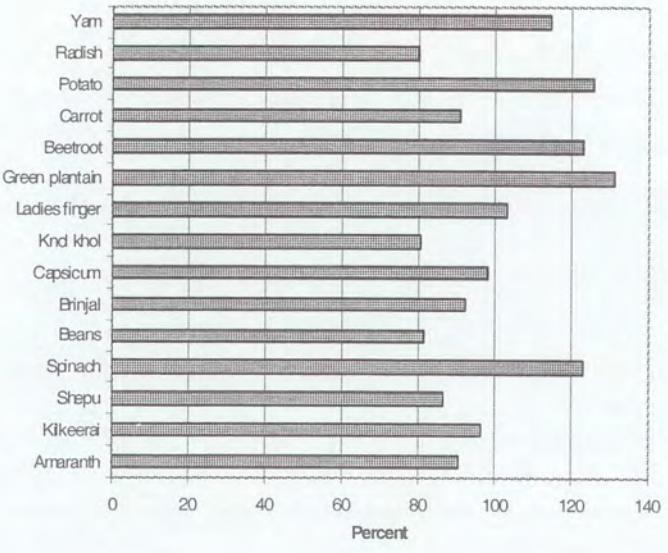
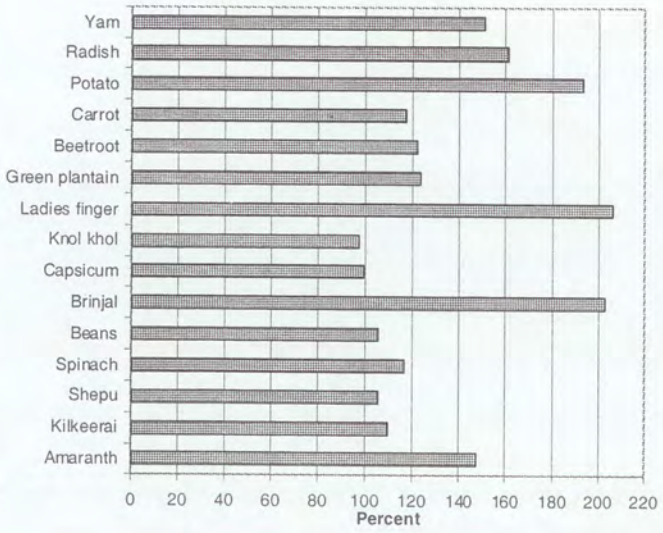


Fig.2. Effect of cooking on soluble fiber content of vegetables in relation to control



Conclusions

It can be concluded that the vegetables analysed in present investigation had fair amounts of dietary fiber which showed slight variations in cooking. However, between cooking methods there were no major differences.

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Effects of Some Flavour Enhancers on Plasma and Tissue Proteins, Lipids and Monoamine Oxidase Activity in Rats

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The effects of flavour enhancers, specifically the Monosodium glutamate (MSG) banded Vedan and A-one, on plasma and tissue proteins, lipids and monoamine oxidase (MAO) activity in rats were investigated. Sixty Sprague-Dawley albino rats were collected and divided into four groups. The first group was fed 0.1% (w/w) Vedan-rat chow and water, second group was fed 0.2% (w/w) Vedan rat-chow and water, third group was fed 0.1% (w/w) A-one-rat chow and water, while the fourth group was fed rat chow and water, ad libitum for 30 days. Feed intake, water intake and body weights of rats were measured daily. The concentration of protein and cholesterol and triglyceride, in the plasma, brain, liver of rats and the brain monoamine oxidase activity were determined. Result of the study indicate that MSG in the flavour enhancers potentiate body weight, feed and water intake of rat, as compared with control rats. The concentration of plasma and tissue proteins and triglyceride significantly ($p<0.01$) increased, while a decrease in cholesterol level was reported in both the Vedan – or A-one fed rats. In addition, these flavour enhancers do not significantly affect brain monoamine oxidase activity in rats. It could be inferred that MSG when consumed at recommended dosage stimulates appetite, promote growth and protein synthesis and may not affect brain biogenic amines or neurotransmitters metabolism.

Keywords: Flavour enhancers, Monosodium glutamate, Plasma, Tissue proteins, Lipids, Monoamine oxidase activity , Rats

Introduction

The Chinese have used certain seaweed (*Laminaria japonica*) to enhance the flavour of food for about two thousand years. In 1908, the flavour –enhancing agent present in the seaweed was identified as glutamic acid (Choi, 1998; Bellisle *et al.*, 1991). Today, the glutamic acid component of the food additive, monosodium glutamate (MSG) is generally synthesized by a method referred to as microbial fermentation (Choi, 1998; Schaumburg *et al.*, 1969). MSG has been used as a flavour enhancer for over a century. It is a free amino acid salt with one sodium atom attached to glutamate (MSG , 2007).

Glutamic acid is an acidic amino acid and one of the most common amino acids found in nature. Glutamate is an excitatory amino acid neurotransmitter, that is , a chemical messenger that triggers the nerve cells to fire(Schwar & Meldrum,1985; Scher & Scher,1992). It is the main component of many proteins, peptides and is present in most tissues. Virtually every food, such as meat, fish, milk and some vegetables contain glutamate. Glutamate is also produced by the body and plays an essential role in human metabolism (Anderson, 1998; Fuke & Shimizu, 1993; Schwarz & Meldrum, 1985; Choi, 1998; Schaumburg *et al.*, 1969). It has been reported that MSG kills brain cells and causes neuro-endocrine disorders in laboratory animals, and causes adverse reactions in humans.

MSG has various detrimental effects, which include dizziness, allergic reactions, fatigue, triggering asthma attacks and exacerbating migraine, headaches (Allen *et al.*, 1987; Raif *et al.*, 2000). In more serious cases ,MSG may even cause neuronal death due to over stimulation and bring about symptoms of the Chinese Restaurant Syndrome (CRS) (Scher & Scher, 1992).It is also linked to disease such as obesity Type 2 diabetes and Alzheimer's disease (Bellisle, 1999; MSG, 2007; Nagata *et al.*, 2006).

Research in the role of glutamate – a group of chemicals that includes MSG in the nervous system also has raised questions about the chemical safety. A 1995 report from the Federation of American Societies for Experimental Biology (FAEB), an independent body of scientists, re-affirm the United States Food and Drug Administration's (FDA) belief that MSG and related substances, are safe food ingredients for most people when eaten at customary levels (Kerr *et al.*, 1979; FDA, 1994). In United States, FDA has classified MSG as generally regarded as safe(GRAS) (Raif *et al.*, 2000).

In Nigeria , the commonest flavour enhancers are Maggi , Knorr , Vedan and A-one .The present study is aimed to ascertain the effect of the consumption of these flavour enhncers ,specifically Vedan and A-one on plasma and tissue proteins ,lipids and monoamine oxidase activity(MAO) in rats. Brain MAO is a metabolizable enzyme of biogenic neurotransmitters , such as serotonin ,catecholamine ,etc.The information obtained will be useful by extrapolation on any possible effect of MSG on neural function .

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There is very scanty information from the wealth of literature on the effect of flavour enhancer on plasma and tissue proteins, lipids and MAO activity. Therefore, the aim of the present study is to ascertain the effects of flavour enhancers on plasma and tissue protein, lipids and monoamine oxidase activity in rats. The information obtained from the study would be useful in rectifying any fear or skepticism often expressed *viz a viz* the usage and safety of MSG for human consumption by extrapolation using animal model.

Materials and Methods

Flavour Enhancers and Source

The brand names of the MSG – Flavour enhances used for the study are Vedan and A-one, recently manufactured by Marc and Mei (Nig.) Ltd., Apapa, Lagos, Nigeria. The batch numbers of the 454g packed sachet were 01692 and 01468 respectively. It was purchased from the reputable Ojuwoye market in Mushin, Lagos, Nigeria .

Formulation of Diets

Diet was 0.1% (w/w) Vedan-rat chow, which consists of 5g Vedan in 5kg rat chow uniformity mixed. Diet 2 was 0.2% (w/w) Vedan – rat chow, consisting of 10g Vedan uniformly mixed with 5kg rat chow, and while Diet 3 was 0.1% A-one –at chow, containing 5g A-one uniformly mixed with 5kg rat chow. Diet 4 was the control diet, which was the commercial rat chow purchased at Pfizer Livestock Feeds (Nig.) Ltd., Ikeja, Lagos, Nigeria.

Sixty virgin Sprague-Dawley male rats weighing 96.9±0.57g were collected from the Animal centre of the College of Medicine of the University of Lagos, Lagos. They were put in groups of three rats per cage and kept in a room with a temperature of 28 ±2^{0}C, and illuminated for 12h per day (0700-1900h). The rats were fed commercial rat chow containing 21% protein with water *ad libitum* for 14 days for acclimatization.

The rats were then divided into four groups containing 15 rats each. The first group of rats was fed 0.1% (w/w) Vedan – rat chow and water, the second group were fed 0.2% (w/w) Vedan – rat chow and water, the third group fed 0.1% (w/w) A-one-rat chow, while the fourth group was fed chow and water, *ad libitum* for 30days. Feed and water intake and body weights of rats were measured daily.

Blood and Tissue Collection

After 30days of feeding the rats with the respective diets, they were starved overnight and then sacrificed by decapitation. Blood samples were collected between 0830h and 0930h by cardiac puncture, with a needle and syringes into plastic tubes containing ethylene diamine tetra acetate (EDTA) disodium salt. The blood was centrifuged at 3000g for 10min at 4^{0}C, and the supernatant, was collected and used for further analysis. After the rats were decapitated, the brains and livers were quickly excised and weighed, and then stored at 20^{0}C for further analysis. There was no detectable loss in monoamine oxidase activity at this temperature.

Determination of Protein determination

The method of Lowry *et al.*, (1951) was used to determined the concentration of protein in the plasma, brain and liver. 0.4ml of ice-cold 3.5% trichloroacetic acid (TCA) (w/w) in 0.125M phosphate buffer, pH 7.4, was mixed with 0.1ml of plasma and the solution centrifuged at 3000g for 10 min at 4^{0}C. The precipitate was dissolved in 90mM Tris – 2.5mM EDTA – acetic acid buffer, pH 7.4, and its protein content determined according to Lowry *et al.* (1951).

Two gram each of brain and liver tissues was homogenized in 9ml of ice-cold 3.5% TCA (w/v) in 0.125M phosphate buffer, pH 7.4 containing 5% sodium metabisulfite and 10% EDTA. After centrifugation at 4800g for 10min at 4^{0}C, the precipitate was dissolved as previously described, and its protein content determined according to Lowry *et al.*, (1951).

Determination of Cholesterol and Triglyceride concentrations

A Randox Cholesterol Kit with Cat. No. CH_20_2 was used for the determination of Cholesterol concentration in the plasma, brain and liver of rats. The cholesterol is determined after enzymatic hydrolysis and oxidation (Tietz, 1976). The indicator quinoneimine is formed from hydrogen perioxide and 4-amino antipyrine in the presence of phenol and peroxidase. The method is called the enzymatic point method. 10μl of the plasma, brain and liver samples were mixed with 1ml of the reagent and incubated for 5min at 37^{0}C. The absorbance of sample and standard against reagent blank was read in a spectrophotometer at 546nm. The concentration of cholesterol was then computed. The concentrations of triglyceride in the plasma, and liver of rats were determined using a Randox triglyceride kit with cat. No TR 217.

The triglycerides are determine after enzymatic hydrolysis with lipases. The indicator is a quinoneimine formed from hydrogen-peroxide, 4-amino phenazone and 4-chlorophenol under the catalytic influence of perioxidase (Tietz, 1976). 10μl of plasma, brain and liver samples were mixed with 1ml of the reagent and incubated for 5 min at 37^{0}C. The absorbance of the sample and standard against reagent blank was read in a spectrophotometer at 540nm. The concentration of triglyceride was then computed.

Determination of brain monoamine oxidase activity

The method of Catrovas *et al.* (1977) was adopted to prepare the enzyme systems of MAO in rat brain, while method of Weissbach *et al.* (1960) and Ebuehi *et al.* (1997) were used to determine brain MAO activity.

Statistical Analysis

Data were subjected to analysis of variance; significant differences were further tested by the Duncan's test (Snedecor & Cochran, 1969).

Results and Discussion

The mean body weight of rats fed 0.1% Vedan –rat chow, 0.2% Vedan-rat chow, 0.1% A-one –rat chow, and rat chow

and water for 30 days were 3.06±0.25 , 3.27±0.41 , 2.96±0.38 and 2.41±0.37 g/rat/day. Results of the study showed that the body weight of rat fed either Vedan – rat chow or A-one-rat chow significantly ($p<0.05$)higher than in the control rats fed rat chow. However ,there was no significant difference in the mean body weight gain of rats fed Vedan or A-one –rat chow diet for 30 days.

The mean feed intake of rats fed 0.1% Vedan –rat chow, 0.2% Vedan-rat chow, 0.1% A-one –rat chow, and rat chow and water for 30 days were 0.63±0.072, 0.68±0.054, 0.60±0.048 and 0.46±0.038 g/rat/day. Results of the study showed that the mean feed intake of rat fed either Vedan – rat chow or A-one-rat chow significantly ($p<0.05$)higher than in the control rats fed rat chow. However ,there was no significant difference in the mean feed intake of rats fed Vedan or A-one –rat chow diet for 30 days.
days.

The mean water intake of rats fed 0.1% Vedan –rat chow, 0.2% Vedan-rat chow, 0.1% A-one –rat chow, and rat chow and water for 30 days were 0.54±0.032, 0.57±0.046, 0.51±0.064 and 0.44± 0.053 ml/rat/day. Results of the study showed that the mean water intake of rat fed either Vedan – rat chow or A-one-rat chow significantly ($p<0.05$)higher than in the control rats fed rat chow. However ,there was no significant difference in the mean water intake of rats fed Vedan or A-one –rat chow diet for 30 days.

The concentration of protein in the plasma and tissue of rats fed different flavour enhancers for 30 days is shown in Table 1. Plasma protein and liver protein concentration significantly ($p<0.01$) increased in the Vedan or A-one fed rat as compared to the controls. The concentration of cholesterol in the plasma, brain and liver of rats fed diets containing flavour enhancers and control diet is shown in Table 2. The concentrations of triglyceride in plasma and liver of rats fed flavour enhancers and control diet are presented in Table 3. Plasma brain and liver triglyceride levels were not significantly altered by the flavour enhancers. Although, rats fed 0.2% Vedan – rat chow significantly showed an increase in the plasma, brain and liver triglyceride concentrations.

Results of the specific activity of brain MAO in rats fed flavour enhancers showed no significant difference when compared to the controls (Table 4).

Data obtained in the present study indicate that consumption of flavour enhancers for 30 days significantly enhanced body growth of the rats, as compared with the control rats. The increase in the body weight of rats fed flavour enhancers such as Vedan and A-one also culminated in the increase in the feed and water intake of the rats fed Vedan or A-one as compared with the control. It could be suggested that these flavour enhancers or promoted palatability so that more food is consumed and therefore a profitable ingredient in the food industry. stimulate appetite. In addition, there was marked hyperactivity in the behavioural changes of the rats fed flavour enhancers for 30days as compared with the controls apart from increase in body weight, feed and water intake. This finding agrees with Bellisle *et al.* (1991). They reported that addition of MSG can lead to increased intake of many

Table 1 : Concentration of protein in the plasma, brain and liver of rats fed diets containing flavour enhancers and control for 30days[1,2]

Diet	*Plasma (mg/100ml)*	*Brain (mg/g)*	*Liver (mg/g)*
0.1% Vedan –Rat chow	1.25 ± 0.004^a	0.665 ± 0.005^a	0.025 ± 0.004^a
0.2% Vedan-Rat chow	1.98 ± 0.006^b	0.725 ± 0.011^b	0.032 ± 0.003^b
0.1% A-one-Rat chow	1.22 ± 0.005^a	0.645 ± 0.006^a	0.018 ± 0.005^c
Rat chow	1.03 ± 0.004^c	0.620 ± 0.002^a	0.016 ± 0.004^c

[1]*Data are expressed as Mean ± S.D. of five determinations.*
[2]*Values carrying different superscripts vertically are significantly ($p<0.01$) different.*

Table 2 : Concentration of cholesterol in the plasma, brain and liver of rats fed diets containing flavour enhancers and control for 30 days[1,2]

Diet	*Plasma (mg/100ml)*	*Brain (mg/g)*	*Liver (mg/g)*
0.1% Vedan –Rat chow	160.55 ± 16.34^a	38.51 ± 1.49^a	24.33 ± 3.10^a
0.2% Vedan-Rat chow	150.64 ± 12.11^a	27.45 ± 4.11^b	19.10 ± 2.30^b
0.1% A-one-Rat chow	162.05 ± 19.04^a	34.47 ± 2.81^a	25.77 ± 2.14^a
Rat chow	179.79 ± 11.23^b	36.25 ± 4.17^a	30.16 ± 2.65^a

[1]*Data are expressed as Mean ± S.D. of five determinations.*
[2]*Values carrying different superscripts vertically are significantly ($p<0.01$) different.*

Table 3 : Concentration of triglyceride in plasma and liver of rats fed diets containing flavour enhancers and control for 30days[1,2]

Diet	*Plasma (mg/100ml)*	*Brain (mg/g)*	*Liver (mg/g)*
0.1% Vedan –Rat chow	120.20 ± 9.43[a]	254.84±23.45[a]	81.49 ±7.05[a]
0.2% Vedan-Rat chow	157.614 ± 14.35[b]	114.72 ±16.11[b]	344.82 ± 31.06[b]
0.1% A-one-Rat chow	117.73 ± 12.06[a]	87.12 ± 7.94[c]	245.43 ± 26.75[c]
Rat chow	113.79 ± 17.24[a]	78.82 ± 9.03[d]	221.07 ± 16.07[d]

[1]Data are expressed as Mean ± S.D. of five determinations.
2Values carrying different superscripts vertically are significantly ($p<0.01$) different.

Table 4 : Changes in specific activity of brain monoamine oxidase of rats fed diets containing flavour enhancers and control for 30days[1,2]

Time of Incubation (min)	*0.1% Vedan-Rat Chow*	*0.2% Vedan-Rat Chow*	*0.1% A-one Rat Chow*	*Rat Chow*
10	0.4357 ±0.0064[a]	0.4537±0.0049[a]	0.4537±0.0008[a]	0.4637±0.0004[a]
20	0.2127 ±0.0052[b]	0.2056 ±0.0004[b]	0.2025±0.0007[b]	0.2279±0.0006[b]
30	0.0601±0.0076[c]	0.0725 ± 0.0005[c]	0.0729±0.0005[c]	0.0601±0.0007[c]

[1]Data are expressed as Mean ± S.D. of five determinations as micromoles/60min/mg protein.
[2]Values carrying different superscripts vertically are significantly ($p<0.01$) different.

foods on first exposure and when added on repeated occasions.

Bellisle *et al.* (1991) reported that MSG is a palatability agent, which has been shown to influence food intake beyond the ingestion of food. The effect of MSG on food intake in rats appears to be dependent on the route of administration. It is apparent that oral administration of MSG gave rise to increase in food intake than in sub-cutaneous administration. (Reddy *et al.*, 1986). The differences may be due to the blood brain barrier system.

Data obtained from the present study for the concentrations of protein, cholesterol and triglyceride in rats fed these flavour enhancers, 0.1% Vedan – rat chow or 0.1% A-one-raw chow, indicate that it potentiated plasma and liver protein levels, but decreased plasma and liver cholesterol levels, while plasma and liver triglyceride levels were not altered. In addition, the concentration of protein, cholesterol and triglyceride in the brain were not significantly affected. This may be due to brain sparing phenomenon (Pardridge, 1988).

However, there is death of information in the literatures on the effect of flavour enhancers in protein and lipid metabolism. It could be suggested in this study that these flavour enhancers may interact or alter the transport or synthesis of proteins and lipids in the body. Further studies are needed to proffer any.

Brain monoamine oxidase specific activities of rats fed flavour enhancers are not significantly altered. This suggest that these flavour enhancers may not stimulate or inhibit brain MAO activity in rats. Since MAO is a major degradative enzyme of the biogenic amines, such as serotonin and catecholamines, one could suggest that Vedan or A-one when consumed at recommended levels may not affect the metabolism of the neurotransmitters.

Ebuehi *et al.* (1999) had previously reported the effect of caffeine and ethanol on the metabolism of 5-hydroxy tryptamine or serotonin, and concluded that brain MAO activity was inhibited by both caffeine and ethanol in the rat. Their findings further suggest that caffeine and ethanol consumption may be detrimental in the regulation of brain function mediated by the serotoninergic functions.

Monosodium glutamate (MSG), the salt of a natural amino acid, glutamic acid, is also much used because it brings out the flavour of the meat, stews, meat pies, and so on, hence, it is an ingredient of many sauces.

Flavour enhancers or seasonings predominantly used by Nigerians in the preparation of food, include cubed seasonings, such as Maggi, Knorr, Doyin and as well as non-cubed seasonings such as Vedan and A-one, commonly called white seasonings. However, the choice of which seasoning to use, the cubed or non-cubed, vary from individual to individual.The United States of America Food and Drug Administration (FDA) classified MSG as a "Generally recognized as safe" or GRAS substance, along with many other common food ingredients, such as salt, vinegar and baking powder. Since 1970, FDA has sponsored extensive reviews on the safety of MSG, other glutamates and hydrolyzed proteins, as part of an on-going review of safety data on GRAS substances used in processed foods. (FDA, 1994). The use of MSG has become controversial in the past 30 years because of reports of adverse reactions in people who have eaten foods that contain MSG. Research on the role of

glutamate in the nervous system has also raised questions about its safety (Kenney & Tidhalls, 1972).

Any glutamate in the food, whether bound in protein or free, or added, is converted in the intestine into free glutamate, and used for energy production by the intestine. Glutamate is also used in the brain as a neurotransmitter. However, the blood brain barrier which controls what type of molecules can enter the brain, does not allow its passage. Therefore, the brain has to synthesize its own glutamate from glucose and other amino acids. Due to the central position of glutamate in metabolism, it has important functions such as substrate for protein synthesis, precursor of glutamine, nitrogen transport, and so on (MSG, 2007; Nagata *et al.*, 2006 ; Bellisle, 1999).

Conclusions

In conclusion, there flavour enhancers, specifically white seasonings such as Vedan or A-One used in the present study significantly increase plasma and liver protein concentrations, decrease plasma and liver cholesterol levels, but do not alter brain proteins, cholesterol and triglyceride levels. In addition these flavour enhancers do not affect brain monoamine oxidase activity in rats. However, from the wealth of literature, there is little or no report on whether flavour enhancers affect plasma and tissue proteins, lipids and brain MAO activity. Therefore, information obtained from the present study will be useful in both nutrition and neurochemistry.

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Effect of Processing Methods on the Removal of Toxic and Antinutritional Constituents of *Jatropha* Meal: A Potential Protein Source

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The Jatropha curcas seeds of commercial variety grown in Karnataka (India) contained 65% kernel and 35% hulls. The kernel was rich in oil (56.5%), protein (20.5%) and the hulls contained very high fiber (54%) and carbohydrates. The seeds were ghani pressed and defatted to obtain meal. Jatropha meal was subjected to different processing methods such as chemical (2% Ca (OH)$_2$, 2% and 4% NaOH) treatments and organic solvent (methanol, ethanol, acetone, chloroform) extractions to remove the toxic constituents. The effect of these treatments on the reduction of antinutritional and toxic constituents was compared with the untreated meal. The chemical treatments inactivated the trypsin inhibitor activity by 80-85% and reduced the tannins (51%), phytic acid (14%) and saponins (44%). While the organic solvents reduced tannins and saponins by 34 and 70% respectively. The toxic phorbol esters were removed to the extent of 89% and 75% by the chemical treatment and solvent extraction respectively. The protein content of the solvent extracted meal was increased from 21 to 42% and improved protein digestibility of the meal. The Jatropha protein was rich in arginine and glutamic acid and the amino acid composition was comparable to FAO reference protein except lysine and sulfur containing amino acids.

Keywords: Processing, Jatropha meal, Phorbol ester, Antinutritional factors

Introduction

Jatropha curcas (*Euphorbiaceae*) popularly called Ratanjyot is a small tree bearing oilseed grown on wastelands or hedges in India and tropical America (Makkar *et al.*, 1997 & 1998). *Jatropha curcas* is grown to fence the farmlands. It is a quick growing and non-palatable to browsing animals. The plant is adaptable to extreme growing conditions, tolerates high temperature and is resistant to drought. The seeds contain 30-32% protein and 60-66% oil (Aderibigbe *et al.*, 1997). The oil is used for lighting, as a lubricant, for making soaps, and as varnish. It can also be used as fuel for diesel engines because it has characteristics close to those of diesel (Gubitz *et al.*, 1999). The cake left after oil extraction is presently used as a fertilizer due to the presence of toxic and antinutritional components such as phytates, saponins, tannins, amylase inhibitors, trypsin inhibitors, glucosinolates, phorbol esters, cyanogens and lectins in the kernel and the cake (Rakshit & Madhvi, 2005). However, the protein rich cake could be used as a potential livestock feed after detoxification (Rakshit & Bhagya, 2006; Chivandi *et al.*, 2004; Aregheore *et al.*, 2003).

The production of Jatropha seed in India is around 1 lakh MT (SARC, 2004). National Oilseed and Vegetable Oil Development Board (NOVOD), Ministry of Agriculture, Government of India has taken up state wise Jatropha plantation on 2.2 - 2.6 million hectare land. In the near future the cake after oil extraction will be available in plenty. At present, not much information is available on the utilization of cake in feed formulation. Therefore, the objective of the present study was to reduce the toxic phorbol esters and antinutritional constituents in the *Jatropha curcas* meal by various treatments to use in feed formulations.

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Materials and methods

Materials

Jatropha curcas seeds of the commercial variety were procured from a local vendor, Karnataka, India. Tannic acid was purchased from Loba Chemicals Pvt. Ltd., Bombay and phytic acid, trypsin, BAPA (benzoyl-DL Arginine-p-Nitroanilide hydrochloride), Tris (Tris hydroxyl methyl amino methane), alpha amylase, pepsin, pancreatin were purchased from Sigma Chemical Company. All other chemicals are of analytical grade.

Preparation of defatted Ghani pressed meal

The pre-cleaned seeds (30 kg) were mechanically pressed using power Ghani to extract the oil. The resulting cake was solvent extracted with hexane in a ratio of 1:5 (w/v) using stainless steel columns. The extraction was repeated 4-5 times, with a soaking time of 12 h each. The fat content of the cake was less than 1g/100g (AOAC, 2000). The cake was air dried, powdered and passed through 22-mesh sieve. The resulting meal was stored at room temperature for analysis. The material balance is given in Fig. 1.

Detoxification/Processing procedures

Treatment with Alkali

Defatted Ghani pressed meal (200 gm) was treated with 2% Calcium hydroxide, $Ca(OH)_2$, 2% and 4% Sodium hydroxide (NaOH). Alkali was added in a ratio of 1:1 (w/v), mixed well till it become a thick paste, and covered with aluminium foil and kept for 30 min at room temperature. The material was

autoclaved at 121°C for 30 min. The samples were dispersed in water in a ratio 1:5 (w/v) and kept for 1 h and filtered through a muslin cloth. The residue was dried at 90°C, powdered and passed through a 60-mesh sieve for analysis.

Treatment with solvent

Defatted Ghani pressed meal (200 gm) were weighed into a 1 litre conical flask. Solvents such as chloroform, ethanol, methanol and acetone were added in a ratio of 1:5 (w/v) and extracted after shaking on a mechanical shaker for 24 h. The solvents were recovered by filtration. The residue was dried, powdered and passed through a 60-mesh sieve for analysis.

Chemical composition

The Jatropha seeds were analyzed by standard method (934.01, 988.05, 920.39, 942.05 and 962.09) for moisture, protein (N x 6.25), fat, ash and crude fiber, respectively (AOAC, 2000).

Toxic and antinutritional components

Phytic acid

The procedure of Thompson & Erdman (1982) was used for phytic acid estimation by converting it to ferric phytate and the phosphorus content was analyzed by the method of Tausky & Shorr (1953). Phytic acid content was derived from phytate phosphorus values by multiplication using a factor of 3.55.

Tannins

Tannin was estimated colorimetrically according to the procedure of Ranganna (1995). The tannin content was expressed as percentage of tannic acid equivalents.

Cyanogenic glucosides

The sample (100 gm) was extracted with orthophosphric acid to produce cyanohydrins, which rapidly decompose to cyanide ion in alkali. Excess pH 6 buffer was added, followed by chloramine-T and pyridine/ barbituric acid to produce a purple color solution which was measured spectrophotometrically at 583 nm. KCN was used as standard (Bradbury *et al.*, 1991).

Trypsin inhibitor activity

This was determined according to the procedure of Kakade *et al.* (1974) using benzoyl-DL-arginine p-nitroanilide hydrochloride as substrate and 2x crystallized bovine trypsin. Results are expressed as trypsin inhibitor units (TIU) in milligram of sample.

Identification of Phorbol esters

Defatted meal (50 gm) was extracted with methanol: water in a ratio of 9:1 (w/v). Extraction was repeated three times. The combined extracts were concentrated in a rotary evaporator and dispersed in diethyl ether, transferred into a separating funnel and then washed with water. The water layer (lower) was discarded. Washing was repeated 3 times. The ether layer was concentrated and used for identification of phorbol esters by TLC, which were coated with silica gel G, 0.5 mm thickness and dried at 105°C for 1 h. The toxic ether fraction was spotted along with groundnut oil and Jatropha oil as reference. Plates were developed in hexane-diethyl ether – acetic acid (80:20:1.25 v/v). Spots with Rf value of 0.05 were scraped from TLC, extracted with methanol and absorption spectra taken scanning in the range of 190 to 300 nm (Gandhi *et al.*, 1995).

Quantification of Phorbol esters

The pooled spots with λ max at 205 and 272 nm were extracted with methanol quantitatively. HPLC was carried out using Waters Symmetry 300™, C_{18} 5µm, 4.6x150mm i.d., column was controlled at 25°C, flow rate 1mL/min, with Waters 1525 HPLC binary pump, Waters 2996 photodiode array detector and millennium software. The solvent used: (A) 1.75 ml of 0-phosphoric acid (85%) in 1 litre of distilled water; (B) acetonitrile. All solvents were degassed by ultrasonification and application of vacuum. The gradient used was as follows: 60% A and 40% B at start, 40% -50% B in 10 min, 50% - 75% B in 30 min, 75% to 100% B in the next 15 min. (Makkar *et al.*, 1997). After the run, the column was washed with tetrahydrofuran. Phorbol esters (4 peaks) appeared between 42 and 51 min (Makkar *et al.*, 1998). The peaks were integrated at 280 nm and the results are expressed as equivalents to phorbol ester – 12 – myristate 13- acetate (obtained from ICN Biomedicals), which appeared at 51 min. Each analysis was conducted in duplicate.

Saponins

This was determined according to the modified method of Gestetner *et al.* (1966).

Haemagglutinins (lectins)

Lectin activity was determined by serial dilution technique, using trypsinized rabbit blood erythrocytes. The highest dilution of the extract, causing visible agglutination, was identified as the titre value (Lis & Sharon, 1972).

Glucosinolates

Glucosinolate content was determined according to the method of AOAC (1984).

Amylase inhibitor activity

This was determined using porcine pancreatic α-amylase according to the method of Worthington (1973). One amylase unit was defined as one µmol of maltose released per min at 37°C at pH 6.9. The maltose released was estimated according to the method of Miller (1959). Amylase inhibitor activity was determined as above and defined as decrease of one amylase unit. Results were calculated and expressed as AIU/gm sample.

Invitro digestibility

The method of Akeson & Stahmann (1964) using pepsin and pancreatin enzymes was employed to determine *in-vitro* digestibility.

Amino acid analysis

Samples containing 5 mg protein were hydrolyzed for 24 h under vaccum at 110°C using 5.8 mol/lit HCl. Amino acid

analysis was carried out by precolumn derivatization using phenylisothiocyanate. The phenyl thiocarbomyl amino acids were analyzed using a Waters Pico-Tag amino acid analysis system (Bidlingmeyer *et al.*, 1984). Tryptophan was estimated by the acid ninhydrin method (Pinter & Molnar, 1990).

Statistical analysis

All the experiments were carried out in triplicate. The experimental data were statistically analyzed by Duncan's new multiple range test to determine the significant differences in each treatment ($P< 0.05$) (Steel & Torrie, 1980).

Results and Discussion

Jatropha seeds were cracked; the hull and kernel were separated and used for chemical analysis. The kernel to shell ratio was 65:35. Makkar *et al.* (1998) have reported the kernel to shell ratio in the range of 53.9 – 64.2: 34.3-46.1 for different varieties of toxic and non-toxic Jatropha seeds. The values are comparable to those reported values of 62:38 and 62.9:37.1 for Indian Kangara and Nasik varieties respectively (Makkar *et al.*, 1997 & 1998). Ghani pressing method was used to recover oil and the cake was extracted with hexane to obtain a defatted meal (Fig. 1). The chemical composition of Hull, kernel and defatted meal is given in Table 1. Jatropha kernel was mainly composed of lipid (56.5%) and crude protein (23.9%). The hull contains high amounts of fibre (54%) and low amounts of protein (5.2%) indicating poor nutritional value with respect to the protein content. While the defatted ghani pressed Jatropha meal contained 20.5% protein, 9.5% ash, 13% fiber and 50% of carbohydrate (Table 1). The protein, fat, fiber and ash contents were similar to that reported by Makkar *et al.* (1997) for different varieties and comparable to Indian varieties (Kangara and Nasik).

The amino acid composition of ghani pressed Jatropha meal is presented in Table 2. For comparison, the amino acid compositions of toxic and non-toxic varieties are also shown in Table 2. Jatropha meal protein contains higher amounts of arginine (14.56%), glutamic acid (18.58%) and lower amounts of sulfur containing amino acids (2.2%) compared to toxic and nontoxic varieties (Makkar *et al.*, 1998). Higher levels of arginine, aspartic acid and glutamic acid in food are associated with the protection against clogging of arteries and proteins rich in arginine has been shown to reduce the cholesterol levels in rats and humans (Carrol & Kurowska, 1995).

The antinutritional and toxic components present in the ghani pressed meal and processed meals are given in Table 3. The trypsin inhibitor activity of the control and processed meal was 15 TIU/mg sample. Upon treatment with NaOH and $Ca(OH)_2$, TIA was inactivated trypsin inhibitor activity by 80-85%. However, organic solvents did not show any significant reduction in the trypsin inhibitor activity. Makkar *et al.* (1997) have reported trypsin inhibitor activity of 18.4 – 27 units for different varieties of Jatropha seeds. The values obtained for this commercial variety was comparable to the reported value for Indian variety (Makkar *et al.*, 1997 & 1998). Moist heating

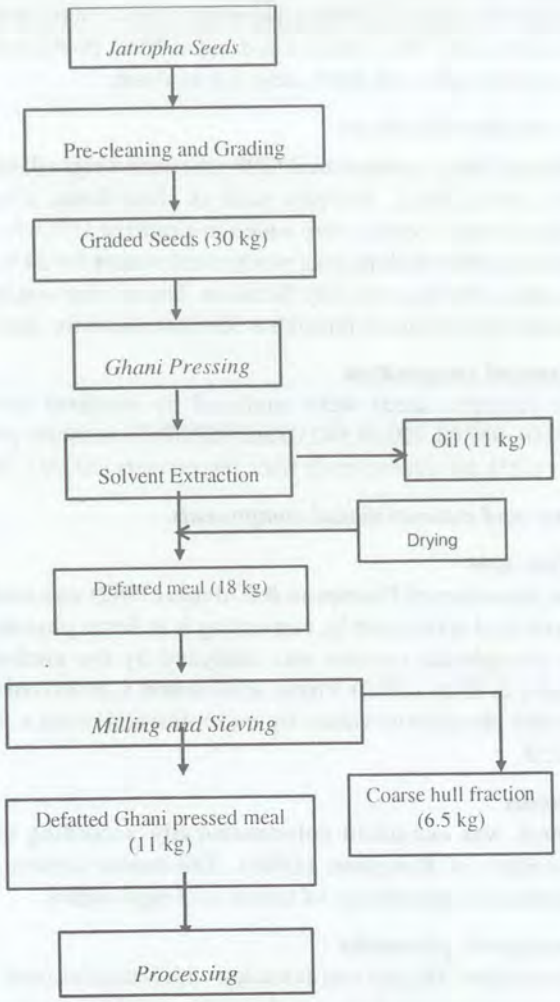

Fig 1: Flow diagram for the preparation of Ghani pressed meal

at 130°C of partially defatted and defatted Jatropha meals for 30 minutes reduced the trypsin inhibitor from 18.9 and 21.3 to 4.5 and 3.8 units respectively (Aderibigbe *et al.*, 1997). Similarly Heat treating the soy proteins at high temperature results in loss of trypsin inhibitors and also change in solubility (Inglett & Victor, 1974). Autoclaving winged bean reduced the trypsin inhibitor activity (Narayana & Narasinga Rao, 1982). Cooking rice bean, mung bean and Pigeon pea reduced the trypsin inhibitor activity up to 76% (Verma & Mehta, 1988; Rani *et al.*, 1996). Anderson (1992) has reported that steaming the soy flour decreased both the trypsin inhibitor activity and nitrogen solubility during the preparation of soy protein concentrate.

The tannin content of untreated meal was 1.03 and it decreased to 0.7, 0.75 and 0.5% after treating the meal with 2% NaOH, 4% NaOH and 2% $Ca(OH)_2$ respectively. The reduction was around 32, 27 and 51% respectively. While in the case of organic solvent extractions the reduction was in the range of 26-34%. Among the treatments, calcium hydroxide treatment followed by soaking and autoclaving were the most

Table 1. Chemical composition of Jatropha kernel, hull and Defatted ghani pressed meal

Constituents (%)	*Kernel*	*Hull*	*Defatted ghani pressed meal*
Moisture	3.9 ± 0.1	9 ± 0.2	7.5 ± 0.2
Protein (N x 6.25)	23.9 ± 0.1	5.2 ± 0.2	20.5 ± 0.1
Fat	56.5 ± 0.5	2.3 ± 0.3	0.8±0.1
Ash	4.0 ± 0.2	5 ± 0.2	9.5 ± 0.5
Crude fiber	4.0 ± 0.2	54.0 ± 1	13.0 ± 0.5
Carbohydrate (by diff.)	7.7	24.5	49.5

Mean ± standard deviation (SD) of three determinations

Table 2. Amino acid composition of Jatropha meal compared to toxic, Non-toxic Mexican varieties and FAO Reference protein (g/100g protein)

Amino Acid	*Ghani pressed meal*	*Toxic variety (Cape Verde) **	*Non-toxic variety (Mexico) **	*FAO Reference Protein ***
Asp	9.94	9.49	9.92	-
Glu	18.58	14.68	15.91	-
Ser	4.53	4.8	4.82	-
Gly	4.81	4.92	4.61	-
His	3.02	3.30	3.08	1.90
Arg	14.56	11.8	12.9	
Thr	2.46	3.96	3.59	3.40
Ala	3.36	5.21	4.94	-
Pro	4.60	4.96	3.80	-
Val	6.28	5.19	5.30	3.50
Met	1.20	1.91	1.76	2.50
Cys	1.00	2.24	1.58	
Iso	5.40	4.53	4.85	2.80
Leu	7.55	6.94	7.50	6.60
Phe	4.39	4.34	4.89	6.30
Tyr	1.97	2.99	3.78	
Lys	4.52	4.28	3.4	5.80
Try*	1.66	1.31	-	1.10

Values are mean of three determinations
*Estimated by Ninhydrin method
* Makkar et al., (1998)
** Data from FAO/WHO/UNO (1985)

Table 3. Effect of processing methods on the removal of toxic and antinutritional factors

Treatment	*Trypsin inhibitor activity (TIU/mg sample)*	*Tannin (%)*	*Phytic acid (%)*	*Saponin (%)*	*Phorbol ester (%)*	*Cyanogenic glucosides (μg %)*
Control	15 ± 0.12^{c}	1.03 ± 0.02^{e}	2.9 ± 0.2^{b}	0.198 ± 0.003^{e}	0.0721 ± 0.0005^{g}	896 ± 10
2% NaOH (30' autoclaved)	2.4 ± 0.17^{a}	0.7 ± 0.04^{c}	2.8 ± 0.15^{b}	0.13 ± 0.004^{d}	0.0194 ± 0.0003^{d}	ND
4% NaOH (30' autoclaved)	2.3 ± 0.15^{a}	0.75 ± 0.03^{cd}	2.49 ± 0.05^{a}	0.088 ± 0.02^{b}	0.0102 ± 0.0006^{b}	ND
2% $Ca(OH)_2$ (30' autoclaved)	3.0 ± 0.23^{a}	0.5 ± 0.03^{a}	2.9 ± 0.2^{b}	0.186 ± 0.01^{e}	0.0079 ± 0.0002^{a}	ND
Chloroform	17.35 ± 0.03^{d}	0.7 ± 0.02^{c}	2.89 ± 0.15^{b}	0.059 ± 0.005^{a}	0.021 ± 0.0007^{e}	ND
Ethanol	14.0 ± 0.2^{b}	0.76 ± 0.1^{d}	2.85 ± 0.12^{b}	0.125 ± 0.01^{d}	0.0178 ± 0.0002^{c}	ND
Acetone	17.75 ± 0.16^{d}	0.70 ± 0.03^{cd}	2.9 ± 0.1^{b}	0.067 ± 0.001^{a}	0.0319 ± 0.0005^{f}	ND
Methanol	19.75 ± 0.01^{e}	0.58 ± 0.03^{b}	2.77 ± 0.18^{ab}	0.110 ± 0.01^{c}	Trace	ND

Mean ± (SD) of three determinations

effective method in reducing the tannins. Aderibigbe *et al.* (1997) have reported the tannin content in the range of 0.2 – 0.4% in different *Jatropha curcas* varieties (Capo Verde and Nicaragua). Makkar *et al.* (1998) reported that tannins were not detectable in the meal of four variety of Jatropha. However, a small amount of tannins were present in shells (2-2.9%). The hulls are known to contain polyphenols. The higher concentration of tannins may be mainly due to the shell, which was crushed during ghani pressing the seeds. Dehulling and washing steps are known to reduce the tannins in mustard seed processing also (Alireza *et al.*, 2006). Processing of rapeseed protein concentrates by successive batch extractions or counter current extractions with water, 70% ethanol; acetone – methanol – water, methanol-aqueous ammonia or acidified methanol reduced the phenolics by 60-67% (Naczk *et al.*, 1998).

The phytic acid content of the control meal was 2.9%. Treating the meal with 2% and 4% NaOH reduced the phytic acid content by 3.5 and 14% respectively, while calcium hydroxide treatment did not reduce the phytic acid content in the meal. However, the organic solvent used for extraction did not reduce the phytate content. The reduction of phytate in the NaOH treated meal was due to leaching out of the phytate during the washing process (Table 3). The values are lower compared to the reported value of 7.2 – 10% for the Mexican varieties of Jatropha meal (Makkar *et al.*, 1998). However, the phytate content was much higher than soybean meal (1.5%) indicating that Jatropha meal can decrease both the bioavailability of minerals and protein digestibility (Reddy & Pierson, 1984).

The saponin content of the control meal was 0.198 %, which was decreased by 70.2, 66 and 44% by extracting with chloroform, acetone and Methanol respectively. However, treating with 2 % NaOH and 4% NaOH reduced the saponin content by 34 and 44 % respectively. The values are lesser than reported by Makkar *et al.* (1997); this may be due to the varietal difference. Saponins of soybean are relatively innocuous, and have also been shown not to be haemolytic (Liener, 1979). Whereas, heat treatment did not have any effect on the saponin content of the Jatropha meal (Aderibigbe *et al.*, 1997; Makkar *et al.*, 1998). However, fermentation has

shown to reduce the saponin content of soybean (Fenwick & Oaken, 1981).

The presence of cyanogenic glucosides in Jatropha meal has been reported by Aderibigbe *et al.* (1997). The ghani pressed meal contained 896 ± 10 µg% of cyanogenic glucosides (Table 3). After treatment with chemicals and organic solvents the cyanogenic glucosides were reduced to undetectable level. Aderibigbe *et al.* (1997) have reported that these cyanogenic compounds are heat labile and their concentration varies with the varieties (Capo Verde and Nicaragua). Similar results have been shown by Makkar *et al.* (1998) in Mexican variety of Jatropha seeds. This may be due to varietal difference.

Identification of phorbol esters

The Phorbol esters identified by TLC with an Rf value of 0.05 was subjected to absorption spectroscopy in the UV range (190-300 nm) showed the absorption maxima at 205.6 and 273.2 nm (Fig. 2). These values were comparable to the values of 202 and 275 nm for *Jatropha curcas* of Indian variety (Gandhi *et al.*, 1995). The results confirm that these compounds are the toxic Phorbol esters.

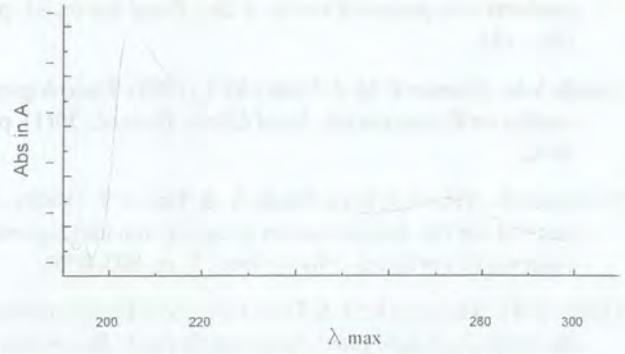

Fig 2: Absorption spectra of Phorbol esters separated by TLC

Quantification of phorbol esters by HPLC

The compound having the λ max at 205.6 and 273.2 nm was quantified by reverse phase HPLC showed 4 peaks between 42-50 min. The standard reference phorbol myristate 13-acetate gave single peak at 51 min., the peaks were integrated and concentration of phorbol esters were calculated and expressed as total phorbol esters (Fig. 3). The phorbol esters content in the control Ghani pressed defatted meal was 0.072%, it was reduced after treating with 2% NaOH, 4% NaOH and 2% $Ca(OH)_2$ to 0.0194, 0.0102 and 0.0079 g % respectively, corresponding to a reduction of 73, 86 and 89%. The results indicate that calcium hydroxide treatment and autoclaving showed higher reduction of phorbol esters than the NaOH treatment. While in the organic solvent extractions, ethanol reduced the phorbol esters by 75%. Among the treatments, 2% $Ca(OH)_2$ and 4% NaOH treatments were effective in reducing the toxic phorbol esters. The values obtained are comparable to those reported for the toxic variety of Capo Verde (Aregheore *et al.*, 2003).

Fig 3: HPLC pattern of Phorbol esters from Jatropha Ghani pressed meal

The protein content of untreated and treated Jatropha meal is given in Table 4. The protein content of the untreated meal was 20.5 %, after treating the meal with 2% calcium hydroxide reduced the protein content to 16%. However, extracting the meal with different solvents increased the protein content to 42%. Extraction with the polar and non-polar solvents has been reported to increase the protein content (Oomah & Mazzog, 1993; Hernandez & Centenoc, 1995).

The protein digestibility of control meal was 75% and it decreased to 68, 50 and 65% respectively for the 2% NaOH, 4% NaOH and 2% Ca $(OH)_2$ treated meals. Whereas, the reduction was in the range of 7-8% in the meals treated with chloroform or ethanol. However, the acetone treated meal showed marginal improvement of 9.5%. Treating castor proteins with calcium hydroxide and autoclaving lowered the protein digestibility value (Puttaraj *et al.*, 1994). The decrease in the digestibility may be due to the interaction of protein with phytate resulting in reduced protein solubility and

Table 4. Effect of different treatments on the protein and *in-vitro* digestibility of protein

Treatment	*Protein (%)*	*In-vitro digestibility (%)*
Control	20.5 ± 0.10^{b}	75.0 ± 0.2^{e}
2% NaOH (30' autoclaved)	24.9 ± 0.10^{d}	67.8 ± 0.5^{c}
4% NaOH (30' autoclaved)	20.9 ± 0.20^{c}	50.3 ± 0.6^{a}
2% Ca $(OH)_2$ (30' autoclaved)	16.3 ± 0.10^{a}	65.8 ± 0.5^{b}
Chloroform	42.0 ± 0.10^{f}	68.8 ± 0.8^{cd}
Ethanol	41.4 ± 0.27^{e}	69.6 ± 0.6^{d}
Acetone	41.3 ± 0.10^{e}	82.2 ± 0.8^{f}
Methanol	41.9 ± 0.10^{f}	74.2 ± 0.4^{e}

Mean ± (SD) of three determinations
Mean values in a same column with different letters differ significant at
$P < 0.05$ by DMRT

inhibiting several enzymes important in digestion (Vaintraub & Bulmaga, 1991). Prolonged heating of proteinaceous foods at alkaline conditions may decrease nutritional availability of proteins by impairing digestibility due to cross linking or recemization (Zdzislaw, 2001).

Conclusions

In conclusion, among the processing methods, 2% $Ca(OH)_2$ treatment was the most effective in reducing the toxic phorbol esters. Jatropha meal with reduced antinutritional constituents; improved protein digestibility and amino acid composition could be a potential source of protein in feed formulations. However, further studies are needed to incorporate Jatropha meal with reduced toxic and antinutritional constituents in feed formulations.

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Growth and Survival of Lactic Acid Bacteria on Acid and Bile Salt Concentrations

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The tolerance of acidic condition and bile salt are the essential criteria for the lactic acid bacteria to be used as probiotic culture. Among the best exopolysaccharide producing lactic acid bacteria, three strains: Lactobacillus rhamnosus YHOC137, L. plantarum NYC30 and L. brevis NVC14, were evaluated for probiotic characteristics based on growth and survival on acidic condition and bile concentrations. None of the strains showed growth at pH 2 and pH 3 and bile salt concentration of 0.1% and above. The survival test showed all the strains can survive pH 3 for longer time but only 0h at pH 2 in significant numbers. Over the time period 1 to 3 h at pH 2 no survival or <103 CFU was observed. Bile concentration of 0.1 % was not fatal to L. rhamnosus YHOC137 and L. plantarum NYC30 in high degree for 2 hours. Survival of L. rhamnosus YHOC137, L. plantarum NYC30 at 0.5 % bile salt for short time was possible and was expected to increase the viability by adaptation to bile containing conditions. The strains were viable in fermented milk at refrigerated storage for 10 days, which indicates those potential probiotics can be stored in refrigerated condition without loss of significant viability. Strain YHOC137 had higher viability over all period. The total reduction of log CFU/ml by 0.43 was not significant (P<0.05) for L. rhanmosus YHOC137 in storage time.

Keywords : Lactic acid bacteria, Acid concentration, Bile salt, Survival

Introduction

Bacteria used as probiotics adjuncts are commonly delivered in a food system and, therefore, are ingested via mouth to the lower intestinal tract. As such, probiotic bacteria should be resistant to conditions of stomach and intestine for their survival. Berada *et al.* (1991) reported the time from entrance to release from the stomach to be 90 min.

Normally healthy stomach has pH below 3.0. Cellular stress begins in the stomach, which has pH as low as 1.5 (Lankaputra & Shah, 1995). During and shortly after a meal, the pH of stomach increase to 3.0 or higher (Martini *et al.*, 1987). After the bacteria passes through the stomach, they enter the upper intestinal tract where bile is secreted into the gut. The concentration of bile is variable and is diluted when mixed with food system (Lankaputra & Shah, 1995). Normal human intestinal tract contains 0.3% bile. This concentration of bile salt has consequently been used as critical concentration in most of the screening assay for probiotic bacteria (Gilliland *et al.*, 1984). Although the resistance mechanisms of bacteria to bile salts are still poorly understood, they have been related in some cases to membrane proteins that either take up (Elkins *et al.*, 2001) or extrude bile salts (Yokota *et al.*, 2000), or produce enzyme, bile salt hydrolase, that transform conjugated bile salt to deconjugated or primary to secondary bile salts (Tanaka *et al.*, 1999).

Probiotic strains must be resistant to the acidity of the stomach, lysozyme, bile, pancreatic enzymes and to antibiotics. These chatacteristics may be observed in vitro and can be used for selection of strains (Salminen *et al.*, 1998). High acidity in the stomach and the high concentration of bile components in the proximal intestine are the first host factors, which affect strain selection and prove the viability of probiotic strains in vivo. Viability is necessary in many of the cases for probiotic activities. However, non-viable probiotic strains perform equally with viable microorganisms in case of lactose intolerance and in the treatment of acute gastro-enteritis (Ouwehand & Salminen, 1998).

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Materials and Methods

Culture preparation

Lactobacillus rhamnosus YHOC137 was isolated from the oral cavity of healthy Chinese adult of Wuxi City. *L. plantarum* NYC 30 was isolated from naturally fermenting yoghurt called *Dahi* collected from the local market of Bhaktapur district of Nepal according to the method given by Badis *et al.*, 2004. *L. brevis* NVC14 was isolated from naturallty fermented vegetables in the Key laboratory of Food Science & Safety, Southern Yangtze University, Wuxi, P.R. China as described by Tamang & Sarker, 1996. The isolated cultures were identified by using API 50 CH Kit (Bio Meriux, France). The working cultures were prepared in MRS broth grown at 37°C for 18 h.

Determination of optimum growth temperature

General purpose growth medium, MRS broth was aseptically inoculated by the fresh active cultures of respective strains, YHOC137, NYC30 and NVC14. The test tubes were incubated at different temperatures ranging from 15- 50°C for 14 h. Growth of the strains were measured by UNICO 2100-UV spectrophotometer (Shanghai Equipment Co. Ltd., China) at 620 nm.

Growth at low pH and in presence of bile salt concentration

10 ml of sterilized MRS broth tubes (pH adjusted to 2 to 8 by 2N HCl) were inoculated by 0.1% of active cultures and were incubated at their respective optimum temperature of growth. After 14 h and 24 h of incubation growth was measured at 620 spectrophotometrically. Similarly, growth of cultures in MRS broth containing ox bile at concentrations of 0.05, 0.1, 0.2, 0.3, 0.4 % were measured. Growth in MRS broth (pH 6.4) served as control.

Tolerance to high acid and high bile salt concentration

The acid and bile tolerance test was done as described by Mishra & Prasad (2005). The acid tolerance of lactobacilli was studied in different pH solutions. The solutions were prepared by adjusting the hydrochloric acid (HCl) solution to pH levels of 1.0, 2.0 and 3.0 in double distilled water. Sterile double distilled water (pH 6.4) served as a control. Solu :tions were sterilized at 121°C for 20 min and stored at room temperature until used. After thorough mixing, 10 ml of each pH solution was taken in sterilized test tubes. A cell suspension of selected cultures containing ca. 10^8 to 10^9 cells/ml was added to each pH solution of 1.0, 2.0 and 3.0 and control (pH 6.4) and mixed. One milliliter from each pH solution was taken immediately (0 h) and after 1, 2, 3 h and serial dilutions were prepared using 0.85% sterile saline. Appropriate dilutions were pour plated in MRS agar and incubated aerobically at corresponding optimum temperatures (at 37°C for *L. rhamnosus* YHOC137 and *L. plantarum* NYC30 and at 30°C for *L. brevis* NVC14) for 72 h.

The bile salt solutions were prepared by using ox bile powder. Sterile double distilled water without ox bile (pH 6.4) was used as control. All solutions were autoclaved and stored at room temperature until used. 10 ml of each solution was taken in sterilized test tubes. A cell suspension of selected cultures containing about 10^8 to 10^9 cells/ml was added to each solution, i.e., 0.05, 0.1, 0.3, 0.5 % and control and incubated at respective optimum temperatures aerobically. One milliliter from each pH solution was taken immediately (0 h) and after 1, 2, 3 h and serial dilutions were prepared using 0.85% sterile saline and pour plated as descried earlier.

Effect of refrigeration storage on viability of potential probiotics in fermented milk

Sterile SKM (11 % w/v) was inoculated by 2% fresh lactic acid cultures each separately and incubated at optimum temperature of growth for 18 h in separate bottles. The coagulum formed was broken by agitation and one milliliter of each culture was serially diluted in 0.85 % sterile saline short after 18 h (0 day). Appropriate dilutions were pour plated in MRS agar and incubated aerobically at corresponding optimum temperatures for 48 h. The rest of the bottles were stored at refrigerated temperature (4°C). Same procedure was followed for 5 days and 10 days storage. The colonies were counted and were expressed as colony forming unit (CFU) per milliliter.

Statistical analysis

All determinations were carried out at least in duplication and mean and standard deviations were calculated.

Results and Discussion

Determination of optimum growth temperature

Fig. 1 shows the optimum growth temperature of three strains of *Lactobacillus* sps. under study. Growth was measured by determining the optical density of growth medium. Optical density is directly proportional to number of cells. However, both viable and dead cell density is measured. Like the published reports, *Lactobacillus plantarum* NYC30 and *L. brevis NVC14* showed optimum growth at 37 and 30°C, respectively. Although *L. rhamnosus* YHOC137 had maximum growth at 35°C the difference in growth at 37°C was insignificant. Most of the mesophilic organisms are grown at 37°C in laboratory work. For technical feasibility, optimum temperature for strain YHOC137 was taken 37 °C and all incubation for growth was carried out at this temperature.

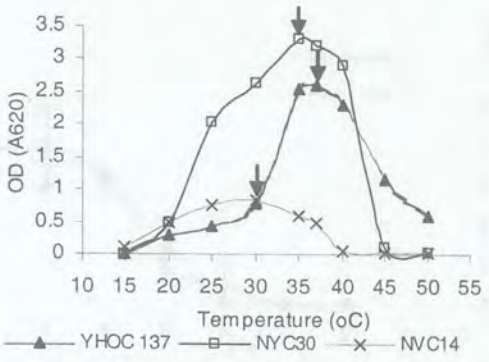

Fig 1: Optimum temperature for growth of *Lactobacillus rhamnosus* YHOC137, *L. plantarum* NYC30 and *L. brevis* NVC14.

Growth at low pH and in presence of bile salt concentration

The growth of *Lactobacilli* sps in MRS broth at pH 2 to 8 was observed for 14 and 24h (Fig. 2). None of the strains showed growth at pH 2 and 3 for any time of period. Strains NYC30 and NVC14 showed little growth at pH 4 and maximum turbidity was developed at pH 6-7. Like temperature, pH is very important factor limiting the growth of bacteria (Meynell & Meynell, 1970). Optimum pH for growth of most lactic acid bacteria is in the range of 5.6-7.4. However, some acid enduring bacteria grow in lower pH than its optimum pH. Proper growth was observed at pH 5 in either case.

Fig. 3 shows the growth of three strains, YHOC137, NYC30 and NVC14 at bile concentration 0.05, 0.1, 0.2, 0.3 and 0.4 % (w/v). None of the strains showed proper growth at 0.1 % and higher concentration of bile. Growth was visible at very low bile containing tubes and in control tube without bile salt. None of the tested strains showed satisfied growth in low pH and bile containing medium. However, all probiotic strains which are capable to tolerate low pH and high concentration of bile salt may not grow at the same pH and bile salt

concentration. Growth at low pH and bile concentration is not necessary to evaluate the organism as probiotic. On the other hand, it leads to extrapolate the increase in number of probiotic strain in situ. The test of growth in such conditions was made to study whether the strains can multiply in stomach and intestine of human and increase in number to assist the beneficial activities.

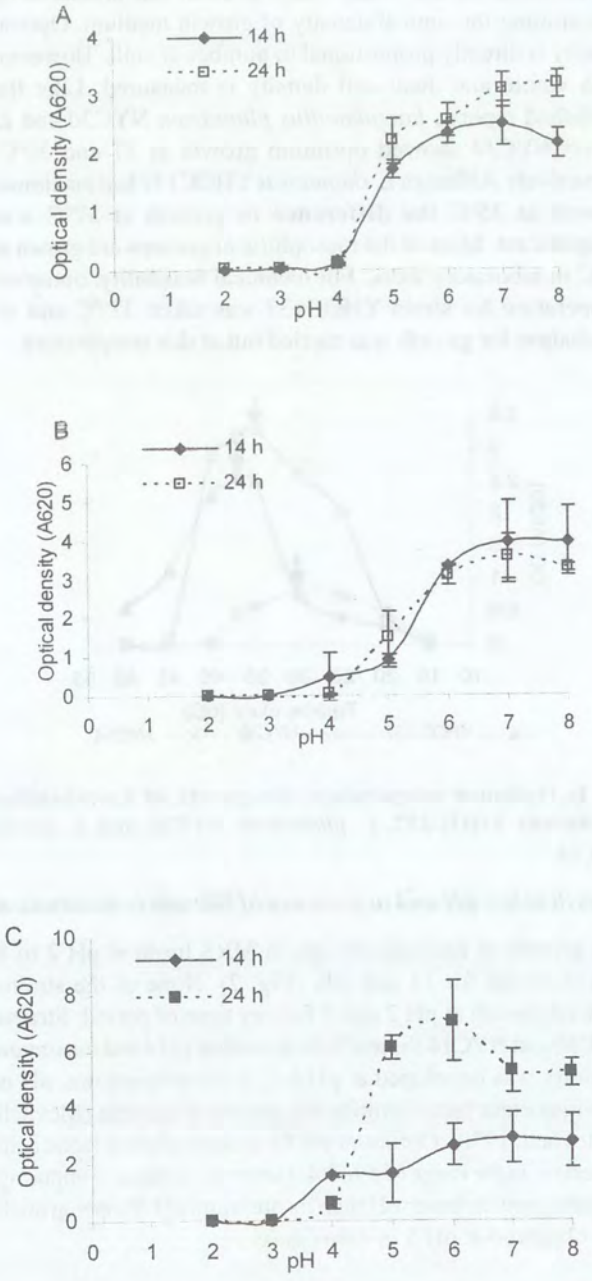

Fig. 2: Growth of LAB in MRS broth at different pH adjusted by 1N HCl. A, *Lactobacillus rhamnosus* YHOC137; B, *L. plantarum* NYC30 and C, *L. brevis* NVC14.

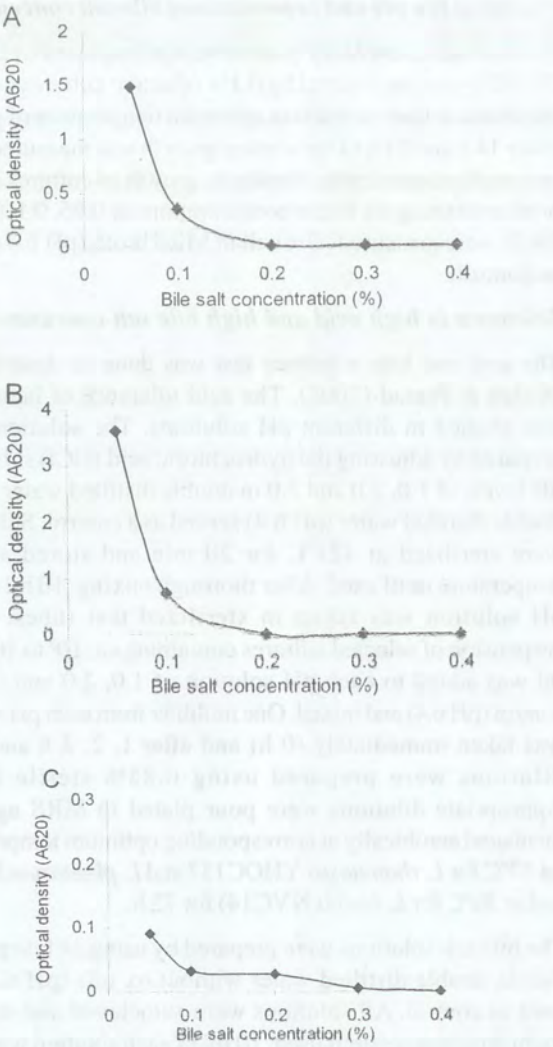

Fig. 3: Growth of LAB in MRS broth containing different bile salt concentration. A, *Lactobacillus rhamnosus* YHOC137; B, *L. plantarum* NYC30 and C, *L. brevis* NVC14.

Tolerance to high acid and high bile salt concentration

Tolerance and survival of lactic acid bacteria were considered an important parameter for probiotic evaluation. Probiotic bacteria need to reach its final destination (Aiba *et al.*, 1998) for competitive exclusion of harmful bacteria. Thus, it is necessary for those to tolerate acid and bile salts (Chou & Weimer, 1999). However, there are many health beneficial properties of probiotic microorganisms where viability is not necessary (Ouwehand & Salminen, 1998). Table 1 gives information about the viability of *Lactobacillus* sps in acidic pH 1, 2, 3 and control pH 6.4 for 0, 1, 2 and 3 h. At pH 1, which is extremely low acidic condition for bacterial survival, none of the strains survived at all. All the strains died immediately (0 h). All the strains survived at pH 2 for 0 h at low degree. Over the time period 1 to 3 h, no survival or $<10^3$ CFU was observed. Survival rate was higher for all strains at pH 3 and was promising but not at pH 2 which is in accordance with

findings by Mishra & Prasad (2005) for tolerance to simulated human stomach pH by strains of *L. casei*. Survival at pH 3 is significant as ingestion with food or dairy products raises the pH in the human stomach to 3.0 or higher (Martini *et al.*, 1987).

Survival of *L. rhamnosus* YHOC137, *L. plantarum* NYC30 and *L. brevis* NVC14 in simulated bile is shown in Table 2. Bile concentration of 0.1 % was not fatal to *L. rhamnosus* YHOC137 and *L. plantarum* NYC30. Although the strains YHOC137 and NYC30 survived at 0.5 % and 0.3 % for 0 h no significant survival was found after 1- 3 h concluding that those strains can not survive more than 0.3% bile salt for longer time. The bile concentration of the human GI tract varies and the mean intestinal bile concentration is believed to be 0.3 % (w/v) (Sjovall, 1959). The tolerance to bile concentration of 0.3 % is considered to be a critical concentration for screening of bile resistant strains (Gilliland *et al.*, 1984) and bile salt tolerance at 0.5% sufficiently proves the viability of strains in harsh conditions of human gastrointestinal tract (Vinderola & Reinheimer, 2003). Survival of *L. rhamnosus* YHOC137, *L. plantarum* NYC30 at 0.5 % bile salt for short time was possible and was expected to increase the viability by adaptation to bile containing conditions or by strategies given by Shah (2000) to improve viability of probiotic organisms.

Table 1: Survival of LAB strains at various pH range over time.

Strains	*pH*	*Time (h)*			
		0	1	2	3
L. rhamnosus YHOC137	1	-	-	-	-
	2	5.2	-	-	-
	3	7.9	5.1	4.9	3.1
	6.4	61	ND	ND	ND
L. plantarum NYC30	1	-	-	-	-
	2	3.8	-	-	-
	3	19.3	2.9	2.0	1.7
	6.4	280	ND	ND	ND
L. brevis NVC14	1	-	-	-	-
	2	1.5	-	-	-
	3	48.5	5.7	0.8	0.7
	6.4	280	ND	ND	ND

The "–" indicates no growth or the CFU value < 9 x 10^3.
Values are mean of two determinations (CFU x 107)

Table 2: Survival of LAB strains at various bile salt concentrations over time. Values are mean of two determinations (CFU x 10^6)

Strains	*Bile (%)*	*Time (h)*			
		0	1	2	3
L. rhamnosus YHOC137	0	29.2	31.4	27.95	20
	0.05	30.32	25.1	21.6	5.4
	0.1	20.6	11.41	0.04	-
	0.3	2.9	-	-	-
	0.5	0.01	-	-	ND
L. plantarum NYC30	0	18.3	13.23	15.9	11.3
	0.05	12.4	13.1	3.9	2.01
	0.1	5.9	0.88	0.56	-
	0.3	1.66	-	-	-
	0.5	-	ND	ND	ND
L. brevis NVC14	0	3.90	3.2	1.8	1.0
	0.05	1.9	2.4	1.5	-
	0.1	-	-	-	-
	0.3	-	-	-	-
	0.5	ND	ND	ND	ND

The "–" indicates negative or the CFU value < 9 x 10^3.

Effect of refrigeration storage on viability of potential probiotics in fermented milk

Fermented milk is the most extensively studied medium for probiotic carrier. Moreover, milk is natural source of very essential nutrients for bacterial growth. Exopolysaccharide production by strains YHOC137, NYC30 and NVC14 was tested in our previous experiments. In this regard milk was chosen to study the viability loss of potential probiotic strains, *L. rhamnosus* YHOC137 and *L. plantarum* NYC30 which are tolerant to low pH and in presence of bile salt, in refrigeration storage over 10 days period (Fig. 4). Strain YHOC137 had higher viability over all period. The total reduction of log CFU/ml by 0.43 was not significant ($P<0.05$). Similarly strains NYC30 and NVC14 had good survival rate.

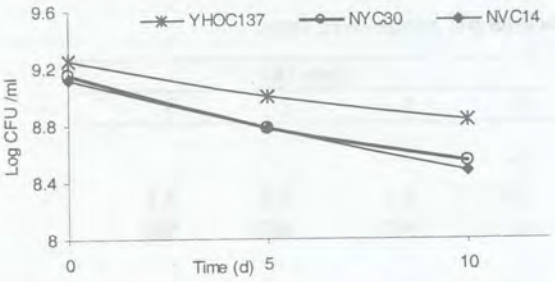

Fig. 4. Viability of lactic acid bacteria in fermented milk over a storage period at 4 °C.

Conclusions

Three strains of lactobacilli which produce high molecular weight exopolysaccharides were subjected to evaluation of probiotic characteristics. The optimum temperature for growth was 37, 37 and 30°C for strains YHOC137, NYC30 and NVC14, respectively. Strains YHOC137 and NYC30 are the isolates from human oral cavity and natural fermented yogurt, respectively, and are tolerant to pH 3 for 3 h and to pH 2 for 0 h only. Bile salt concentration at 0.3 % for short time was tolerated by both strains. Moreover, YHOC137 could survive at 10^4 CFU/ml at 0.5 %. Although the bile tolerance ability of tested strains was not very satisfied those strains can be regarded as potential probiotics. The strains were viable in fermented milk at refrigerated storage for 10 days, which indicates those probiotics can be stored in refrigerated condition without loss of significant viability.

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Effect of Nucleotides Supplementation on the Growth of Bifidobacteria and Pathogenic Bacteria

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Nucleotides account for 2-5% of the non-protein nitrogen fraction of human milk and they have beneficial effects upon the immune system, small intestinal growth and development, hepatic function, and lipid metabolism. Nucleotides also proposed act as growth factors for some strains of bifidobacteria. Therefore, nucleotides supplementation of formulae for potential nutrition is considered beneficial. The present work examined the effect of the in vitro supplementation of nucleotides on the growth of bifidobacteria and pathogenic bacteria, as propose herein the use of a menadione-catalyzed luminol chemiluminescent assay for the rapid determination of viable microorganisms. In the present study it is verified that supplementation of nucleotides increased the growth of bifidobacteria and inhibited the growth of pathogenic bacteria. However, stimulated growth of bifidobacteria showed variable degree according to the several nucleotides added.

Key words: Nucleotides, Bifidobacteria, Pathogenic bacteria, Growth, Viable cells

Introduction

Nucleotides were first isolated from human milk by Deutsch and Nilson in 1960 and 13 acid soluble nucleotides have been identified. Nucleotides belong to the non-protein-nitrogen (NPN) fraction of milk. They account for 2-5% of the NPN fraction of human milk, nonetheless their concentration in human milk decreases with advancing lactation (Janas & Picciano, 1982; Gil & Sanchez-Medina, 1982). The contribution of the NPN fraction to the total nitrogen content of milk is at least three times greater in human milk than in bovine milk. Nucleotides are considered to be dietary non-essential nutrients because they can be derived from de novo synthesis and nucleotide salvage pathways. However, dietary nucleotides could play a role in the developing gastrointestinal tract or can effect on diarrhea disease (Brunser *et al.*, 1994; Uauy *et al.*, 1990), and the known benefits of breastfeeding on infant morbidity have led researchers in infant nutrition to examine the role of nucleotides that may play on the developing immune system of infants (Rudolph *et al.*, 1984; Carver *et al.*, 1991; Yamamoto *et al.*, 1997). Thus, the various effects of the nucleotides have been recognized to be of value especially to preterm and small gestational age (SGA) infants (Cosgrove *et al.*, 1995). On the other hand, it is well known that the intestinal microflora of breast-fed infants is different from that of infants fed with cow's milk formula. Bifidobacteria usually predominate in the gut of breast-fed infants, whereas Gram-negative bacteria are the dominant organisms in infants fed with cow's milk formula (Braun, 1981). *In vitro* studies have shown that nucleotides can act as growth factors for some strains of bifidobacteria, and hence, nucleotide supplementation of formulas for parenteral nutrition is considered beneficial. This study is conducted to examine the effect of the *in vitro* supplementation of nucleotides on the growth of microorganisms including bifidobacteria, and propose herein the use of a menadione-catalyzed luminol chemiluminescent assay for the rapid determination of viable cell.

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Materials and Methods

Strains and reagents

Bifidobacteria, *Clostridium perfringens* and *Escherichia coli* were obtained from JCM (Japan Collection of Microorganisms). Nucleotides were obtained from Yamasa Company (Chiba, Japan). Bactolumix reagent's kit was obtained from ATTO Company (Tokyo, Japan).

Media and growth conditions

Bifidobacteria strains (*B. bifidum* 'JCM 7004', *B. breve* 'JCM 1192^T, *B. longum* 'JCM 1217^T, *B. infantis* 'JCM 1222') were propagated in MRS (Difco, USA) at 37°C under anaerobic conditions. *Clostridium perfringens* 'JCM 1290^T' and *Escherichia coli* 'JCM 1649^T' were propagated in BHI (brain heart Infusion, Oxoid LTD., England) and LB broth at 37°C under anaerobic and aerobic conditions, respectively. M9 medium was used to perform all the growth assays. 5'-monophosphate adenosine·Na (AMP), 5'-monophosphate cytidine·Na (CMP), 5'-monophosphate guanosine·Na (GMP), 5'-monophosphate inosine·Na (IMP), and 5'-monophosphate uridine·Na (UMP) were supplemented (concentration 1 mg mL^{-1}) in M9 medium. All M9 medium samples were inoculated at 1% from an overnight MRS, LB and BHI culture solution.

Determination of viable cell using colony forming units (CFU) method and chemiluminescent assay

A series of 10-fold dilutions of bacterial cell suspension was prepared in 0.1% peptone broth. One ml portion of the dilution was spread on a MRS, LB and BHI agar plate. The colonies

on each plate were counted after 24 h incubation, and the number of viable bacteria per milliliter of each original suspension was calculated as CFU mL^{-1}. On the other hands, the enhancement or the inhibitory effect of bacteria as a result of nucleotides supplementation was determined by using the chemiluminescent assay, i.e. fifty microliters from each of the above diluted cell suspension were placed in a separate tube, and then 50 µl of menadione was added. When the appointed incubation time was finished (37°C for 10 min), luminol chemiluminescent intensity (LCI) was determined after an injection of 100 µl of the chemiluminescent reagent by using luminescener AB-2200 (ATTO, Tokyo).

Statistical analysis

All results presented in this paper represent the mean of triplicate measurements for each assay. The results are expressed as means ± SD, the significance of differences being determined by Student's *t* test.

Results and Discussion

Menadion-catalyzed luminol chemiluminescent assay has been proposed for the rapid determination of bacterial viability. Menadione is considered to be reduced by NAD(P)H. Menadione-oxidoreductase in bacteria, and the active oxygen species as final products are determined by luminol chemiluminescent assay. The count of CFU differs with LCI, because CFU is not the meaning volume of LCI. However, we confirmed that LCI is correlated with the number of CFU (data not shown). Therefore, chemiluminescent assay can determine the growth or inhibition of viable cell by an annexation against control. The present study observed that viable cell is using luminol chemiluminescent upon the addition of luminol to the mixture of menadion and tested bacteria strains.

The effect of AMP, CMP, GMP, IMP and UMP·Na supplementation were varied according to the tested strains. The present study first estimated the ability of tested strains to grow in M9 medium. As shown in Fig. 1, all strains exhibited minimum growth in M9 medium. This result indicated that supplemented nucleotides in M9 medium is expected to provoke the stimulatory or inhibitory effect on the bacterial growth. Therefore, the present work selected M9 medium as a minimum media for the microbial growth. Growth of *B. bifidum* in M9 medium was significantly stimulated ($P < 0.05$) by supplementation with CMP, and UMP, while it was inhibited ($P < 0.05$) by supplementation with AMP (Fig. 2A). The order of the nucleotides based on their stimulatory effect on the bacterial growth were UMP> CMP> IMP> GMP. On the other hand, all of the five tested nucleotides exhibited stimulatory effects on the growth of *B. longum* in the M9 medium (Fig. 2B). The stimulatory effect of such nucleotides on the growth of this strain was in the order of AMP ($P < 0.01$) > GMP ($P < 0.05$) > CMP ($P < 0.05$) > UMP ($P < 0.05$) > IMP ($P < 0.05$). In the case of *B. infantis* (Fig. 2C), stimulatory effect was obtained by the addition of AMP ($P < 0.05$), CMP ($P < 0.05$), GMP ($P < 0.01$) or IMP ($P < 0.05$). However, an inhibitory effect on the growth of these cells was realized when the cells were cultivated in M9 medium in the presence of UMP ($P < 0.01$), and such inhibition was estimated by over three folds (Fig. 2C). The stimulatory effect of supplemented nucleotide on the growth of *B. infantis* was in the order of GMP > IMP > CMP > AMP.

From the above mentioned results, we can verify that almost all of the bifidobacteria were stimulated by supplementation of nucleotides in M9 medium, but the growth of *B. breve* showed various levels of inhibition as a result of addition of the nucleotides to its culture medium (Fig. 2D).

Furthermore, we tested the effect of supplemented nucleotides on the growth of *E. coli* and *C. perfringens* as negative control. It is evident from Fig. 3 that all of the nucleotides added induced inhibitory effect on the growth of *E. coli*, and in particular, GMP and UMP which significantly inhibited ($P < 0.01$) the growth of *such pathogenic organism.* Addition of AMP and IMP also induced more than five-fold inhibition in the growth of *E. coli* in comparison with the control ($P < 0.01$). On the other hand, the effect of added nucleotides against *C. perfringens* was variable (Fig. 4). AMP declared significant inhibitory effect ($P < 0.01$), while the inhibitory effects of both CMP and IMP was not significant ($P > 0.05$). On the contrary, GMP and UMP showed significant increase in *C. perfringens* growth over two-and 1.5-folds, respectively (Fig. 4). The inhibitory effect against *C. perfringens* that induced by the addition of AMP was over 3–folds when compared with the control.

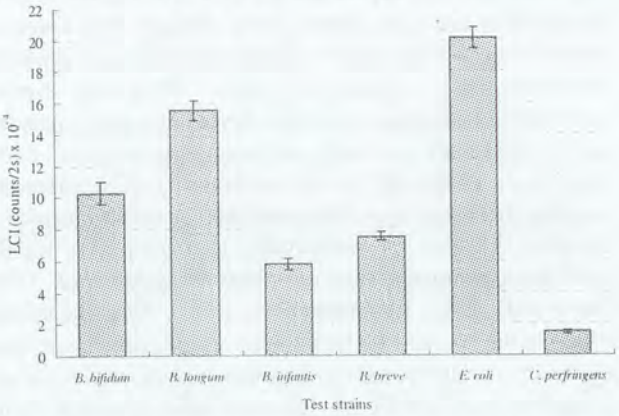

Fig. 1: Comparison of growth between test bacteria obtained after 24 h incubation in minimum nutrition medium as M9 medium. Luminol chemiluminescent inhibition (LCI) is expressed viable cells.

There are variable results about the effect of nucleotides on the microbial growth. Tanaka & Mutai (1980) reported that bifidobacteria growth is enhanced *in vitro* when a selective medium with added nucleic acids is utilized. Similarly, Gil *et al.* (1986) reported stimulatory effects of the nucleotides upon bifidobacteria growth, as they revealed an increase in the enterobacteria growth in the stools of infants who were fed nucleotide supplemented formula. The present study also

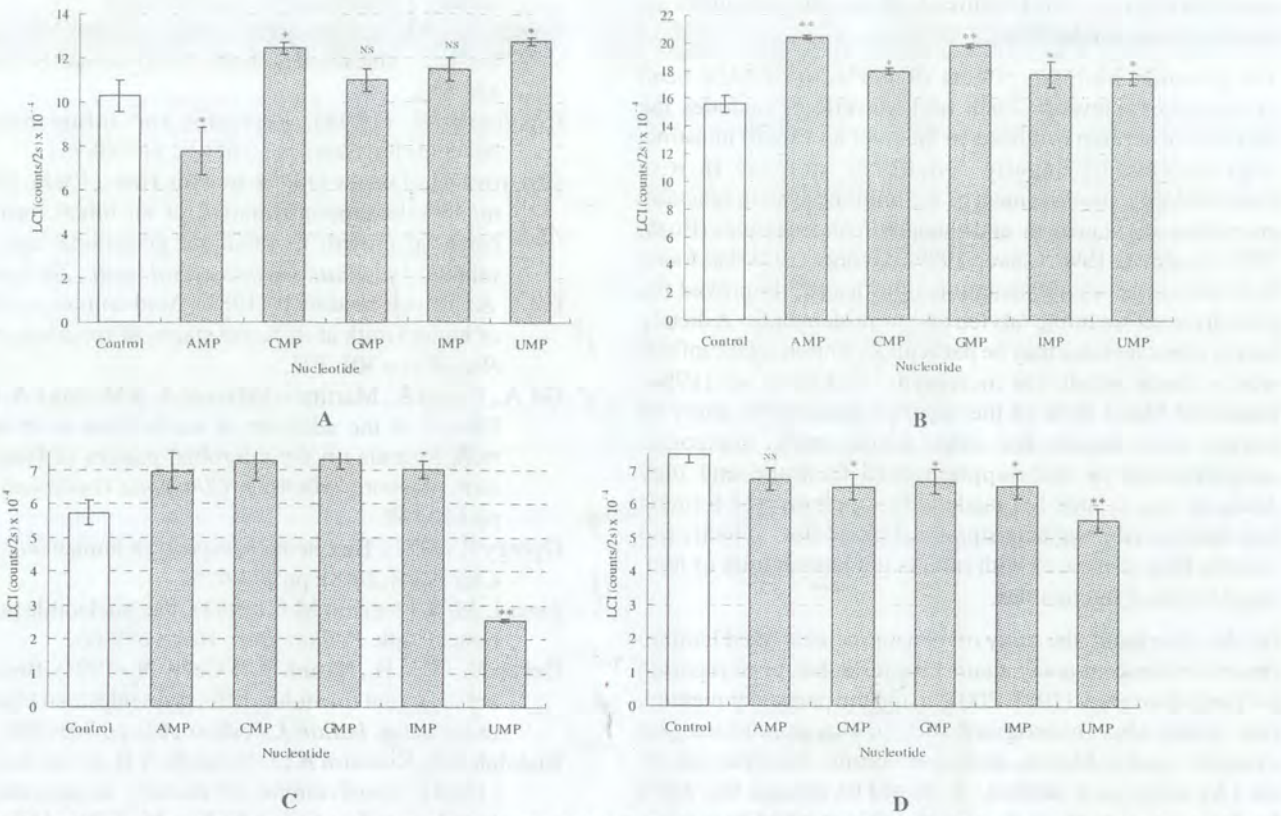

Fig. 2: Effect of supplementation nucleotides on the growth of *Bifidobacterium* (A, *B. bifidum*; B, *B. longum*; C, *B. infantis*; D, *B. breve*) in M9 medium.

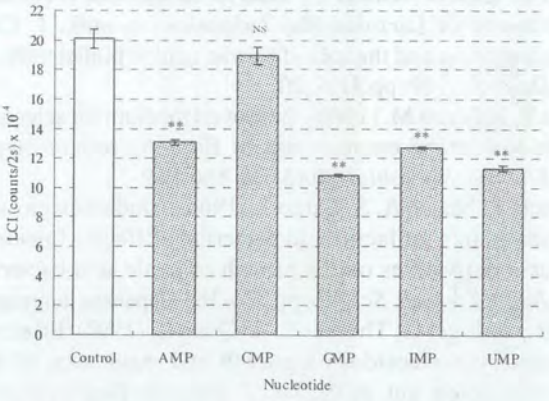

Fig. 3: Effect of supplementation nucleotides on the growth of *E. coli* in M9 medium.

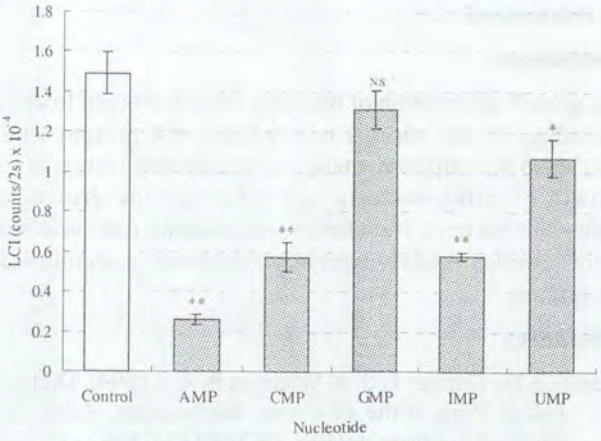

Fig. 4: Effect of supplementation nucleotides on the growth of *C. perfringens* in M9 medium.

confirms the same result as of above research group where in, this study found that the nucleotides enhanced the growth of bifidobacteria except for *B. breve* (Fig. 2). However, Balmer *et al.* (1994) reported that supplementation of the nucleotides had no effect on the growth of bifidobacteria in the faecal flora of infants. Likewise, Brunser *et al.* (1994) indicated that there was no difference in the clinical characteristics of the episodes or in the enteropathogens isolated from symptomatic or asymptomatic infants between the nucleotide-supplemented and non-supplemented infants. On the other hand, Taniguchi *et al.* (1965) reported that adenine, hypoxanthine, inosine, adenosine, and adenine nucleotides stimulated the growth of *L. bulgaricus* in milk, whereas guanine, xanthine, guanosine, and guanine nucleotides inhibited its growth. Suzuki *et al.* (1986) also reported that adenine, guanine, AMP, and GMP stimulated the growth of several *L. bulgaricus* strains, while the effect of such added nucleotides was different with some test strains of *L. bulgaricus*. The results of previous researchers indicated that the effect of supplementation of nucleotides differs according to the bacterial strains. Balmer *et al.* (1994) suggested that the variable effects of dietary nucleotide supplementation is referred to many factors such as the difference in microbiological techniques, test host (babies),

microbiological environment, diet, and amounts of supplemented nucleotides.

The possible biologic effects of nucleotides have been extensively reviewed. Such biologic effects includes the decrease of protein synthesis in the liver and small intestine, improvement of hepatic functions, increase in iron bioavailability, improvement of the immune system function, promotion of gut growth, and changes in plasma lipids (Boza, 1998; Cosgrove, 1998; Carver, 1999). György (1971) has found that nucleotide supplementation significantly improved the growth rate of weanling rats fed on low protein diets. A dietary source of nucleotides may be particularly important for infants whose tissue needs are increased. Özkan *et al.* (1994) measured blood flow of the superior mesenteric artery of healthy term infants fed either human milk, nucleotide supplemented or non-supplemented formula, and they declared that infants fed nucleotide supplemented formula had significantly higher postprandial blood flow velocity and volume flow compared with infants fed human milk or non-supplemented nucleotides.

On the other hand, the study of menadione-catalyzed luminol chemiluminescent assay against bacteria, has been reported by Yamashoji *et al.* (2001, 2004) using the particular method. Our group also investigated the various conditions (for example; media, dilution, menadion volume, bacterial species etc.) by using such method. It should be noticed that MRS broth is not suitable to be used in this method because it includes enzyme inhibitors, while M9 medium could be used for this method.

Conclusions

The growth stimulation of bifidobacteria is thought to differ according to the various nucleotides; the present study concluded that supplementation of nucleotides increased the growth of bifidobacteria and inhibited the growth of pathogenic bacteria. Therefore, supplementation of nucleotide in cow's milk formula is expected to improve the microflora in the infants.

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Organic Acids and Volatile Components in *Masyaura*

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Masyaura, a legume based traditional fermented food product of Nepal, was prepared from blackgram dhal and colocosia tuber by traditional sundrying as well as controlled fermentation and mechanical drying methods. Organic acids and volatile components were analyzed. The results indicated that acetic acid was found to be the major organic acid in Masyaura prepared by traditional sundrying technique. The proportion of acetic acid was observed low in controlled fermented Masyaura with concomitant increase in the lactic and citric acid. Aldehydes, esters, acids, alcohols and pyrazines were detected in Masyaura as prepared by sun-drying as well as controlled fermentation techniques.

Keywords: Organic acids, Volatile components, Fermentation, *Masyaura*, Nepal

Introduction

Masyaura is an important legume based traditional fermented food product of Nepal. It is usually prepared from legumes like Blackgram or Greengram and vegetables like Colocosia or Raddish or Ashgaurd in cottage or home scale level. *Masyaura* is one of the famous savory foods used as adjunct in curry and commonly consumed by all class of people as a substitute of meat (Karki, 1986; Gajurel & Baidya, 1979). *Masyaura* technology is also highly correlated to the technology of preservation of perishable vegetables.

Masyaura, similar to Indian *Wari*, are friable, brittle and spongy dried balls of 2-5 cm diameter. Once it is prepared, the dried balls are stored for future use. At the time of cooking, it is mixed with curry to make soup and served with rice as side dish (Gajurel & Baidya, 1979). Little informations on nutritional, biochemical, antinutritional and bioavailability characteristics of *Masyaura* are available in the literature (Gajurel & Baidya, 1979; Karki, 1986; Dahal *et al.*, 2003a & 2003b; Dahal & Qi, 2005 & 2006). In this study, an attempt has been made to obtain the informations on organic acids and volatile components existing in *Masyaura* since such information is lacking in the literature.

Materials and Methods

Blackgram (*Phaseolus mungo*) dhal was collected from local market of Kathmandu, Nepal and Colocosia (*Colocosia esculanta*) tuber was purchased from the local market of Wuxi, China.

Preparation of Masyaura:

Traditional method: Cleaned split blackgram dhal was washed thoroughly with water to remove husk and foreign matter and soaked for 16h in water. The soaked dhal was lightly hand washed in tape water to remove husk and ground into a thick paste (Moisture around 70%) using a wet grinder. The colocosia tuber was washed peeled and finally wet grinded to get thick paste (Moisture of colocosia paste was maintained about 70% with colocosia powder. Colocosia tuber was sliced, blanched at 70°C for 5 min dried at 50°C and was grinded to get powder). The blackgram dhal paste and colocosia paste was then mixed in a ratio of 1:1. The dough was made into small lumps weighing 20-30g each distributed 1-2 inches apart on steel trays between lumps, left to ferment overnight at ambient temperature. The spongy textured balls are then sun dried for 5 days (Room Temp.20°C, Humidity 70%). *Controlled fermentation and mechanical drying technique*: Dough was prepared and distributed on steel trays similar to traditional method. Trays were then subjected for fermentation at 30°C (RH 80-90%) for 24 h and dried at 50°C for 12-16 h. This fermentation temperature and time was selected based on the sensory characteristics as per the previous study (Dahal & Qi, 2005). The dried products were packed and stored in sealed polythene bags at ambient temperature. The dried *Masyaura* was powdered in a grinder to pass through 60 mesh sieve and used for chemical analysis.

Chemical Analysis:

Organic acid Composition: Organic acid was extracted according to the method of Mulyowidaro *et al.* (1991a) and separated by HPLC according to the method of Mulyowidarso *et al.* (199b) with slight modification. Lactic, citric, acetic, tartaric, malic and ascorbic acid were determined. One g of flour sample was dissolved in 25 ml of deionised water and sonicated for 10 min using sonicator. The content was centrifuged at 5000 rpm/20 min and the supernant was treated with 0.2% trifluroacetic acid to remove protein, allowed to stand for 1 h and again centrifuged. Supernant was filtered to pass through 0.45 μm and separated by HPLC. HPLC condition used was as follows: Mobile phase: 0.1M KH_2PO_4, pH 2.5. Flow rate: 1 ml/min, Column: C_{18} from waters company, Detector: UV at 214nm. Injection volume: 5 μl. The concentration of individual acids was determined by the reference to the elution of standard organic acids at 0.05% of

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individual organic acids. *Volatile components*: Volatile components were determined according to the method of Yang & Peppard (1994) with slight modification following the solid phase micro-extraction technique as follows. About 10 g of sample was kept in a extraction bottle before keeping it at water bath at 70°C for 35 min. The flavor collected in the head space of the bottle was collected in the especial collector syringe used for the purpose and the sample of flavor was injected in the GC-MS instrument (Finnigan Company, USA). The GC condition of the instrument used was as follows. Initial temperature (°C): 40 Initial time (min):1; Rate # 1 (°C/ min):5; Final temperature # 1 (°C):120; Hold time #1 (min):0; Rate #2 (°C /min):8; Final temp #2 (°C):200; Hold time #2 (min):0; Rate #3 (°C /min):12; Final temp #3 (°C):250; Hold time #3 (min):7; Injection temperature (°C): 250; Injection Mode: Splitless; Carrier gas: He; Carrier gas mode: Constant flow; Carrier gas speed (ml/min): 1; Column: DB-5, 50mm * 0.32 mm * 1.0 cm ; Instrument: Finnigan Trace MS. Similarly the MS condition used was as follows: Ionization mode: EI+, Emission current (μA): 200, Electron energy (eV): 70; Interface temp (°C): 250; Source temperature (°C): 200; detection voltage (V): 350.

Results and Discussion

Organic acids in *Masyaura* samples prepared both by traditional as well as controlled fermentation method were analyzed. Lactic, acetic, citric, tartaric, malic and ascorbic acids were analyzed and results are reported in Table 1. Blackgram dhal (acidity 1.0%) contained 0.22% acetic acid, 0.13% malic acid; citric, tartaric and lactic acids were in minor amount. Blackgram paste which was resulted from the soaking, washing and grinding of blackgram dhal (acidity 1.4%) showed 0.29% citric acid and minor amounts of tartaric, malic, ascorbic and lactic acid. Dough mixed with colocosia paste (0.90% acidity) noted 0.33% acetic, 0.33% citric acid and others in minor amount.

Sun-dried *Masyaura* (acidity 1.84%) contained acetic acid as major organic acid accounting 0.91% (Table 1). Controlled fermented *Masyaura* (2.29% acidity) showed 0.67% of acetic acid, 0.35% citric acid and 0.31% lactic acids. Proportion of lactic acid was found to be increased in case of controlled fermentation conditions. Sun-dried samples showed acetic acid as major organic acid (about 50% of total acid) followed by lactic, tartaric and ascorbic acid. Control fermented sample showed acetic acid (29.3%), citric acid (15.3 %) and lactic acid (13.5%) as the main organic acids followed by ascorbic and tartaric acid in minor amounts. Lactic, acetic, citric, pyroglutamic, succinic and pyruvic acids were also reported as major organic acids in *Miso*. During *Miso* fermentation, citric and malic acids decreased while others increased. Citric (44.4mg), succinic (29.7mg), lactic (22.7mg), malic (20.4mg) per 100g are produced during *Koji* fermentation with fumaric, alpha-ketoglutaric acids and volatile acids such as acetic and propionic acid (Ebine, 989). Similarly, the main organic acids detected were acetic, lactic, citric and succinic in *Tapai*-A Malaysian Fermented Food (Merican & Queen-Lan, 1989).

Table 1: Organic acid profile of *Masyaura* and its raw ingredients

Sample	*Tartaric*	*Malic*	*Ascorbic*	*Lactic*	*Acetic*	*Citric*	*Total acidity*[#]
Blackgram dhal	0.03	0.13	ND	0.03	0.22	0.08	1.00±0.05
	(3)	(13)		(3)	(22)	(8)	
Blackgram paste	0.02	0.04	0.04	0.01	ND	0.29	1.40±0.07
	(1.4)	(2.9)	(2.9)	(0.7)		(20.7)	
Mixed dough*	0.03	ND	0.01	0.04	0.33	0.33	0.90±0.04
	(3.3)		(1.1)	(4.4)	(36.7)	(36.7)	
Sun dried *Masyaura*	0.05	ND	0.02	0.05	0.91	ND	1.84±0.09
	(2.7)		(1.1)	(2.7)	(49.6)		
Controlled Fermented *Masyaura*	ND	ND	ND	0.31	0.67	0.35	2.29±0.03
				(13.5)	(29.3)	(15.3)	

- Results are % of organic acids on dry basis. Values in the parenthesis indicates the percentage of total acidity.
- * Dough mixed with colocosia paste.
- # Acidity % lactic acid on db, determined by titrimetric method.
- ND refers to not detected.

Anaerobic fermentation favors higher proportion of lactic acid whereas exposure to air increases the proportion of volatile especially acetic and butyric acid (Onyekwere *et al.*, 1989). Lactic acid was the major organic acid reported in Japanese soy sauce along with pyroglutamic, acetic, levulinic, formic citric and succinic acid (Fukushima,1989)

Volatile components in sun-dried *Masyaura* and controlled fermented *Masyaura* were analyzed.Total of 80 and 96 volatile components were detected in sun-dried and controlled fermented *Masyaura* respectively. Large number (31-55) and amount (23-65%) of hydrocarbons followed by aldehydes, esters, acids, alcohols, pyrazines were detected in *Masyaura* (Table 2). Flavoring components for sun-dried *Masyaura* and controlled fermented *Masyaura* are given in Table 3 and 4 respectively.

Table 2: Volatile components from different *Masyaura*

Volatile component		*Sun-Dried Masyaura*	*Controlled-fermented Masyaura*
Hydrocarbons	N	46	31
	A	51.7	23.4
Aldehydes	N	3	5
	A	3.8	4.9
Esters	N	3	5
	A	2.1	2.3
Acids	N	2	7
	A	7.8	3.4
Alcohols	N	5	9
	A	2.7	6.1
Pyrazines	N	3	1
	A	10.2	0.3
Others	N	18	38
	A	21.7	58.1

[N: Number of volatile components, A: Amount% of total volatile components]

The organoleptic properties of fermented foods are one of the key parameters in distinguishing the product. The organoleptic properties of fermented foods usually differ from those of unfermented substrate and are dependent on biochemical activities of the associated microorganisms. New aroma and flavor compounds are often produced by fermentation including acids, alcohols, carbonyl compounds, esters and pyrazines (Cook, 1994). Fermentation is a process which provides variety of new flavors to enjoy. Flavor may be developed from the primary metabolism of the microorganism or from residual enzymatic activity once the microbial cell has lysed. Esters, acids, alcohols and pyrazines are quite important to the flavor of fermented foods (Heath & Reineccius, 1986; Cook, 1994; Danehy, 1986). Ethanol and its esters were found to be the most important aroma compounds in rice *Miso* as well as barley *Miso*. Isobutyl, butyl and isoamyl alcohol produced by the yeast fermentation are also important to the aroma of well-fermented *Miso* (Ebine, 1989). Lactic acid, acetic acid, ethanol and diacetyl were the most abundant compounds.

Table 3: Volatile Components in Sun-dried *Masyaura*

RT (min)	*Volatile components* *Name*	*Formula*	*M.W.*	*SI*	*RSI*	*Area (%)*
	Hydrocarbons					
10.65	Dodecane	$C_{12}H_{26}$	170	904	936	0.54
11.68	Heptadecane, 2,6,10,15 tetramethyl	$C_{21}H_{44}$	236	825	834	0.67
12.48	Tetradecane	$C_{14}H_{30}$	198	905	905	1.53
13.20	Hexadecane	$C_{16}H_{34}$	226	884	897	0.78
14.35	Hexadecane	$C_{16}H_{34}$	226	904	920	2.34
13.73	Tetradecane, 3 methyl	$C_{15}H_{32}$	212	869	884	0.42
14.88	Docosane	$C_{22}H_{46}$	310	695	731	0.53
15.06	Pentadecane-7-methyl	$C_{16}H_{34}$	226	861	864	0.73
15.18	Pentadecane-5-methyl	$C_{16}H_{34}$	226	837	883	0.42
15.32	Tetradecane, 4,11-dimethyl	$C_{16}H_{34}$	226	854	857	0.74
15.39	Pentadecane, 2 methyl	$C_{16}H_{34}$	226	874	878	1.11
15.61	Pentadecane, 3 methyl	$C_{16}H_{34}$	226	828	887	1.61
16.17	Heptadecane 2 methyl	$C_{18}H_{38}$	254	790	801	0.83
16.23	Hexadecane	$C_{16}H_{34}$	226	931	935	4.04
16.36	n-Nonylcyclohexane	$C_{15}H_{30}$	210	798	878	0.89
16.41	Hexadecane, 2-methyl	$C_{17}H_{36}$	240	692	774	0.45
16.51	Heptadecane, 9 hexyl	$C_{23}H_{48}$	324	644	645	0.49

Conclusions

Overall, acetic acid was found to be the major organic acid in sun-dried *Masyaura* where acetic, citric and lactic acids were found to be the major organic acid in controlled fermented and mechanical dried *Masyaura*. Tartaric, malic and ascorbic acid were found in minor amounts. Aldehydes, esters, acids, alcohols and pyrazine were detected as the major volatile components in *Masyaura* responsible for the flavoring-components.

16.62	Pentadecane,2,6,10 trimethyl	$C_{18}H_{38}$	254	907	910	3.73
16.71	Heptadecane 4-methyl	$C_{18}H_{38}$	254	814	839	0.54
16.89	Undecane, 2,3-dimethyl	$C_{13}H_{28}$	184	773	867	0.91
16.94	3-undecane, 6-methyl (E)	$C_{12}H_{24}$	168	644	718	0.81
17.04	Tetradecane, 4-ethyl	$C_{16}H_{34}$	226	762	814	0.62
17.09	Cyclohexane.1-(cyclohexylmethyl)-4-methyl-cis.	$C_{14}H_{26}$	194	615	656	0.60
17.19	Hexadecane, 4-methyl	C17H36	240	871	905	0.45
17.25	Hexadecane, 2-methyl	C17H36	240	801	830	1.25
17.51	Pentadecane, 2,6,10,14 tetramethyl	$C_{19}H_{40}$	268	882	888	7.75
17.82	Heptadecane, 3 methyl	$C_{18}H_{38}$	254	737	781	0.54
18.08	Heptadecane	$C_{17}H_{36}$	240	919	923	1.90
18.21	Cyclopentane, undecyl	$C_{16}H_{32}$	224	889	896	1.04
18.33	Cyclohexane, decyl	$C_{16}H_{32}$	224	828	884	0.64
18.69	Heptadecane, 8 methyl	$C_{18}H_{38}$	254	803	851	0.67
18.91	9-Nonadecane	$C_{19}H_{38}$	266	653	744	0.48
19.07	Heptadecane, 9 hexyl	$C_{23}H_{48}$	324	775	775	1.19
19.29	Heptadecane, 3 methyl	$C_{18}H_{38}$	254	785	849	1.13
19.42	Hexadecane, 2,6,10,14- tetramethyl	$C_{20}H_{42}$	282	909	913	2.89
19.87	Henecosane	$C_{21}H_{44}$	296	886	906	0.95
20.51	Benzene [1-Pentylhexyl]	$C_{17}H_{28}$	232	821	871	0.53
20.63	Benzene [1-Butyllheptyl]	$C_{17}H_{28}$	232	886	903	0.84
21.39	Benzene, 1-ethylnonyl	$C_{17}H_{28}$	232	810	868	0.46
22.21	Benzene, 1 pentylheptyl	$C_{18}H_{30}$	246	901	908	0.43
22.34	Benzene, 1 Butyloctyl	$C_{18}H_{30}$	246	847	884	0.62
22.45	Benzene 1, methyldecyl	$C_{17}H_{28}$	232	823	866	0.44
22.65	Benzene 1-Propylnonyl	$C_{18}H_{30}$	246	848	866	0.42
28.96	Octacosane	$C_{28}H_{58}$	394	828	885	0.47
32.16	Octacosane	$C_{28}H_{58}$	394	885	915	0.73
35.37	Octacosane	$C_{28}H_{58}$	394	805	882	0.51
	Total					**51.66**
	Aldehydes					
3.89	Butanal 3 methyl	$C_5H_{10}O$	86	845	854	0.52
12.42	Nonanal	$C_9H_{18}O$	142	820	865	0.79
23.83	Tetradecanal	$C_{14}H_{28}O$	212	909	956	2.51
	Total					**3.82**
	Esters					
21.31	Propionic acid, 2 methyl-1(1,1 dimethyl ethyl)-2 methyl, 1,3 propanediyl ester	$C_{16}H_{30}O_4$	286	762	885	0.53
31.51	1,2 Benzenedicarboxylic acid bis [2-methylpropyl] ester	$C_{16}H_{22}O_4$	278	906	913	0.56
36.35	Glycine N,N acetylglycyl butyl ester	$C_{10}H_{18}N_2O_4$	230	464	656	0.99
	Total					**2.08**
	Acids					
13.57	Acetic acid	$C_2H_4O_2$	60	950	955	1.79
33.08	n-Hexadecane acid	$C_{16}H_{32}O_2$	256	883	894	6.06
	Total					**7.85**

	Alcohols					
21.91	1 octadecanol	$C_{18}H_{38}O$	270	814	844	0.52
22.87	1-Undecanol	$C_{11}H_{24}O$	172	892	935	0.48
26.18	1-Pentadecanol	$C_{15}H_{32}O$	228	748	909	0.53
35.79	Benzene ethanol, 4-hydroxy	$C_8H_{10}O_2$	138	823	895	0.46
36.27	Ethanol,2[9,12-octadecadienyloxyl)	$C_{20}H_{38}O_2$	310	718	731	0.68
	Total					**2.67**
	Pyrazines					
11.62	Pyrazine 2,3 dimethyl	$C_6H_8N_2$	108	842	890	0.48
12.72	Pyrazine trimethyl	$C_7H_{10}N_2$	122	874	914	0.80
14.06	Pyrazine tetramethyl	$C_8H_{12}N_2$	136	853	854	8.94
	Total					**10.22**
	Other compounds					
4.87	2,3-Butanedione	$C_4H_6O_2$	86	846	853	0.77
6.11	Methanamine N-N dimethyl N-oxide	C_3H_9NO	75	484	757	0.61
10.45	1,3 dioxolane, 4,4,5-trimethyl-2 Pentadecyl	$C_{21}H_{42}O_2$	326	351	385	1.43
14.24	Cycloheptasiloxane tetradecamethyl	$C_{14}H_{42}O_7Si_7$	518	787	796	0.67
14.76	2,3,5 trimethyl-6-ethylpyrazine	$C_9H_{14}N_2$	150	841	878	0.48
15.24	2,3 butanediol, S-CR.R	$C_4H_{10}O_2$	90	888	899	0.53
15.84	1 methoxy-2propylacetate	$C_6H_{12}O_3$	132	671	739	1.78
15.93	2,3 -Butanediol	$C_4H_{10}O_2$	90	791	739	8.21
16.77	Spiro-10-tricyclo[5.5.0.0(5,9)]decane-7,8 diol-2-oxirane,1 methyl 4-isopropyl (8s)	$C_{15}H_{24}O_3$	252	443	459	0.40
17.39	Cyclooctasiloxane, hexadecamethyl	$C_{16}H_{48}O_8Si_8$	592	790	820	0.86
17.95	2-Pyrolidione, 1-methyl	C_5H_9NO	99	878	924	1.41
20.81	3-[6,6-dimethyl-5-oxohept-2-enyl]-cyclohexane	$C_{15}H_{24}O_2$	236	580	642	0.38
20.91	5,9-undecadien-2-one,6,10-dimethyl	$C_{13}H_{22}O$	194	752	778	1.35
23.15	Benzene, 1 ethyldecyl	$C_{18}H_{32}O$	264	749	814	0.40
25.39	Cedryl Propylether	$C_{18}H_{32}O$	264	761	764	1.01
29.12	Diethyl phthalate	$C_{12}H_{14}O_4$	222	900	907	0.41
33.74	Phenol, nonyl	$C_{15}H_{24}O$	220	871	874	0.50
33.88	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	948	952	0.46
	Total					**21.66**

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Table 4: Volatile components in controlled fermented *Masyaura*

RT min	*Flavor Name*	*Formula*	*MW*	*SI*	*RSI*	*Area (%)*
	Hydrocarbons					
12.05	Toluene	C_7H_8	92	858	868	0.08
15.11	Ethylbenzene	C_8H_{10}	106	735	847	0.10
16.13	P Xylene	C_8H_{10}	106	801	837	0.51
16.05	Undecane, 2, 7- dimethyl	$C_{13}H_{28}$	184	660	794	0.40
15.67	Octadecane, 4, 14-dibutyl	$C_{26}H_{54}$	336	451	456	0.14
16.43	2-Ethyl-3-methyl cyclopentane	C_8H_{14}	110	539	694	0.65
16.92	Nonane, 3-methyl	$C_{10}H_{22}$	142	855	909	0.27
17.60	Nonane-4-methyl	$C_{10}H_{22}$	142	801	923	2.46
17.89	Octane, 4-ethyl	$C_{10}H_{22}$	142	743	846	1.18
18.58	Decane	$C_{10}H_{22}$	142	764	765	5.18
18.76	Cyclohexane,1,2-diethyl, cis	$C_{10}H_{20}$	140	495	635	0.44
19.23	Octane, 3,5-dimethyl	$C_{10}H_{22}$	142	860	890	0.57
19.40	Decane, 2,9-dimethyl	$C_{12}H_{26}$	170	824	882	0.24
19.50	Nonane, 3,7-dimethyl	$C_{11}H_{24}$	156	891	896	0.32
19.84	Nonane 2,5 dimethyl	$C_{11}H_{24}$	156	831	832	1.22
19.92	Decane-4-methyl	$C_{11}H_{24}$	156	890	903	0.96
20.02	Decane-2-methyl	$C_{11}H_{24}$	156	816	821	1.39
20.17	Decane-3-methyl	$C_{11}H_{24}$	156	817	832	1.19
20.76	Undecane	$C_{11}H_{24}$	156	714	719	3.16
20.87	Benzene,1-methyl-4-[1-methelethenyl]	$C_{10}H_{12}$	132	605	842	0.14
21.08	2,6-Dimethyldecane	$C_{12}H_{26}$	170	703	821	0.34
21.30	Decane, 3,7-dimethyl	$C_{12}H_{26}$	170	822	860	0.16
23.69	Decane, 2,3,5 -trimethyl	$C_{13}H_{28}$	184	458	776	0.08
21.57	Decane, 2,6,7 trimethyl	$C_{13}H_{28}$	184	796	822	0.19
24.30	Dodecane	$C_{12}H_{26}$	170	827	916	0.09
25.82	Tetradecane	$C_{14}H_{30}$	198	787	854	0.13
26.84	1H-cyclopropleazulene,decahydro.1,1,7-trimethyl-4-methelene	$C_{15}H_{24}$	204	843	886	0.44
27.02	Decahydro-4,4,8,9,10-Pentamethylnaphthalene	$C_{15}H_{28}$	208	461	675	0.10
19.00	3-cyclohexane-1-acetic acid			533	707	0.28
19.09	Nonane, 2,6-dimethyl			907	911	0.88
26.15	Perhydrophenalene	$C_{13}H_{22}$	178	744	813	0.08
	Total					**23.37**
	Aldehyde					
11.77	Heptanal	$C_7H_{14}O$	114	742	780	0.16
12.94	Hexanal	$C_6H_{12}O$	100	555	567	2.03
14.63	2-Hexanal	$C_6H_{10}O$	98	834	842	1.56
22.83	Decanal	$C_{10}H_{20}O$	156	899	919	0.22
18.02	Benzaldehyde	C_7H_6O	106	789	836	0.90
	Total					**4.87**
	Alcohol					
8.65	Butanol, 3-methyl	$C_5H_{10}O$	86	791	830	0.80
11.84	2-Penten-1-ol	$C_5H_{10}O$	86	722	772	0.16
14.88	2-Hexano-1-ole	$C_6H_{12}O$	100	793	827	0.32
14.98	1 Hexanol	$C_6H_{14}O$	102	821	829	1.21
16.56	3-undecanol-3-ethyl	$C_{13}H_{28}O$	200	440	507	1.36
14.53	3-Furan methanol	$C_5H_6O_2$	98	648	724	0.82
19.58	Benzyl alcohol	C_7H_80	108	942	948	0.71
20.92	Nonanol	$C_9H_{18}O$	142	885	923	0.48
21.35	Phenylethyl alcohol	$C_8H_{10}O$	122	799	879	0.27
	Total					**6.13**
	Ester					
34.35	Hexanedioc acid, dioctyl ester	$C_{22}H_{42}O_4$	370	688	707	0.52
10.93	Cyanic acid, 2 methylpropyl ester	C_5H_9NO	99	718	850	1.35
18.71	Acetic acid, hexyl ester	$C_8H_{16}O_2$	144	781	812	0.13

23.20	Propanoic acid, octyl ester	$C_{11}H_{22}O_2$	186	590	709	0.14
29.92	Hexenedioic acid, bis(2-ethoxyethyl)ester	$C_{14}H_{26}O_2$	290	566	588	0.15
	Total					**2.29**
	Acids					
9.16	Propionic acid	$C_3H_6O_2$	74	813	868	0.37
11.98	Butanoic acid	$C_4H_8O_2$	88	775	817	0.07
13.62	Butanoic acid, 3-methyl	$C_5H_{10}O_2$	102	888	894	0.63
13.91	Butanoic acid, 2 methyl	$C_5H_{10}O_2$	102	763	809	0.30
15.97	3-Bromo-3 methylbutyric acid	$C_5H_9BrO_2$	180	541	598	0.84
21.22	6-oxo-3-thio-octanoic acid	$C_6H_{12}O_3S$	164	507	606	0.09
21.79	Octanoic acid	$C_8H_{16}O_2$	144	644	741	1.11
	Total					**3.41**
	Pyrazine					
20.61	Pyrazine-tertamethyl	$C_8H_{12}N_2$	136	655	735	0.29
	Total					**0.29**
	Other compounds					
7.82	2-2 methylene bis [3,4,6-trichloroanisole]	$C_{15}H_{10}C_{16}O_2$	432	314	361	24.51
8.90	1 Nitro 2 acetamido-1,2, dideoxy-d-mannitol	$C_8H_{16}N_2O_7$	252	491	537	0.50
9.32	1-1 Dichloro-3-3-dimethylbutane	$C_6H_{12}C_{12}$	154	541	589	0.49
9.82	Furan, 2 ethyl	C_6H_8O	96	928	932	1.07
10.06	2-butanone, 3-hydroxy	$C_4H_8O_2$	88	823	828	0.85
11.51	Desmethyldeprenyl	$C_{12}H_{15}N$	173	468	705	0.23
12.32	Benzeneacetamide, N-methyl	$C_9H_{11}NO$	149	349	820	10.71
12.68	Propane, 2,2-dimethylbxy	$C_5H_{12}O_2$	104	548	618	8.56
13.23	1,3-Bis trimethylsilyl benzene	$C_{12}H_{22}Si_2$	222	517	563	0.14
13.80	1,3,2-dithiaborole,2-ethyl	$C_4H_9BS_2$	132	443	510	0.39
14.04	Furfural	$C_5H_4O_2$	96	669	813	0.13
15.22	Methyl methoxy-hydroxymethyl-amine	$C_3H_9NO_2$	91	653	720	0.27
15.36	Hexanenitrile	$C_6H_{11}N$	97	693	748	0.34
16.30	3-Buten-2-one	C_4H_6O	70	495	827	0.09
16.71	15-Crown-5	$C_{10}H_{20}O_5$	220	595	603	1.65
17.13	Ethyl-1-oxo-2-oxabicyclo[3,3,0] octane-8-carboxylate	$C_{10}H_{14}O_4$	198	441	763	0.18
17.71	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	296	553	708	1.01
18.24	5-Heptane-2-one, 6-methyl	$C_8H_{14}O$	126	537	673	0.08
18.43	Furan 2 pentyl	$C_9H_{14}O$	138	814	819	0.96
18.90	Cyclohexane, isocyanato	$C_7H_{11}NO$	125	624	682	0.49
19.65	2-Pyrrolidione, 1-methyl	C_5H_9NO	99	827	895	1.27
20.43	Acetophenone	C_8H_8O	120	805	905	0.11
22.93	Tricyclo [7, 2, 0, 0(2, 6)] undecan-5-ol, 2, 6, 10, 10 tetramethyl isomer 1.	$C_{15}H_{26}O$	222	569	579	0.08
23.11	7b-formyl-8a-ethoxy-trans-bicyclo (4,3,0) non-3-ene	$C_{12}H_{18}O_2$	194	379	512	0.08
23.50	Hydrazine,2[difluro(1 methylpropyl)silyl 1,1-dimethyl	$C_6H_{16}F_2N_2Si$	182	367	544	0.11
23.90	Cyclotetrasiloxane, octamethyl	$C_8H_{24}O_4Si_4$	296	792	881	0.20
26.20	Amodiaquine TMS derivative	$C_{23}H_{30}ClN_3O_{Si}$	427	506	601	0.16
26.42	Methoprene	$C_{19}H_{34}O_3$	310	535	580	0.10
26.61	Cyclohexanamide, N-cyclohexylidene	$C_{12}H_{21}N$	179	674	681	0.43
27.13	Polyneuridine oxindole, dehydroxymethyl	$C_{20}H_{22}N_2O_3$	338	302	342	0.17
27.21	1-propanone-1,2,6-dimethyl-4-propoxyphenyl-3-1piperidyl	$C_{19}H_{29}NO_2$	303	619	639	0.19
27.26	Cyclopropanemethanol-2-methyl-2-4-methyl-3-pentenyl	$C_{11}H_{20}O$	168	610	718	0.11
27.43	Picrotoxinin	$C_{15}H_{16}O_6$	192	389	399	0.08
28.64	Cyclooctasiloxane, hexadecamethyl	$C_{16}H_{48}O_8Si_8$	592	580	627	0.10
28.74	2-E. Hexanoic acid (45)-amino-5-methyl	$C_7H_{13}NO_2$	143	750	771	0.15
28.92	N-cyclohexyl-2-pyrrolidone	$C_{10}H_{17}NO$	167	846	849	1.44
32.15	9-octadecanamide (2)	$C_{18}H_{35}NO$	281	841	842	0.73
32.58	9-octadecanamide	$C_{18}H_{35}NO$	281	594	637	0.24
	Total					**58.1**

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Quality Assessment of Packaged Drinking Water Commercially Available in Kathmandu Valley

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Sales of packaged or bottled water in Nepal have been increasing in recent years, largely as a result of public perception of purity and safety, its convenience, scarcity of adequate public water supplies and chemically unfit under ground water to drink in some regions. In this study, altogether 65 bottles and jars of 55 brands of packaged drinking water i.e. natural spring or mineral water and processed or purified water were analyzed for chemical and microbiological quality assessment. The result shows that 38 samples were within the quality standard. The reason of substandard was high iron and ammonia content content, low pH, high total mesophilic count and presence of fecal coliform and E. coli. Radiation activity was detected in 41 samples. Chemically 22 samples and microbiologically 27 samples were out of Nepal Standard. Arsenic was found in seven samples exceeding WHO guideline values.

Keywords: Packaged Water, Quality, Radiation Activity, Microbial & Chemical Characteristics.

Introduction

Safety access of drinking water is an essential to health, a basic right and essential component of effective policy for minimum health protection. WHO has reported that about 30,000 people and children die everyday from water related diseases, more critically, in developing or least developing countries (WHO, 2006). According to the data published by Department of Public Health, Government of Nepal, about 3500 children are dying due to water borne diseases (DPH, 2006). Depending on the climate, physical activity and culture, the drinking water need for individuals vary but it is estimated to be about 2 liters per day for a 60 kg person and one liter per day for a 10 kg child (FAO/WHO, 2003; WHO, 2004).

The chemical and radiological parameters have important role in source selection and as a whole, selection of the brands of packaged water in market (CAC, 1985; Dux, 1996).When the radiation activity exceeds the limiting value, it should trigger further investigation in to the radio nuclides responsible and the possible risk taken into total account local circumstances. Similarly, the greatest microbial risk is associated with ingestion of water that is contaminated with human or animals (including birds) fecal and other pathological organisms like cyanobacteria (Dufour, 2003; WHO/FAO, 2003).Moreover, It is more difficult to manage packaged drinking water because it is stored in plastic container for longer periods and higher temperature in the containers or bottles that are reused with out adequate cleaning or sterilization (IPCS, 2000; WHO, 2004).

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The attitude of most of the urban consumer is to purchase packaged or bottled drinking water, may be, due to its taste, convenience or fashion, influence from foreigners and international media, awareness to safety and potential health benefits (Gleick, 2004; WHO, 2004), and scarcity of adequate public water supplies as well as chemically unfit underground water to drink in some regions. Some consumers believe that Natural mineral water have medicinal properties or offer other health benefits. Such waters are typically of high mineral content and consumer has culture to accept it as food rather than drinking water per se (Gleick, 2004; CAC, 1997). Forty eight packaged water industries are registered in and licensed by Department of Food Technology and Quality Control (DFTQC) producing sixty-five brands of packaged or bottled waters that are available in local market of Kathmandu valley, in two forms i.e. mineral or natural spring water and processed or purified drinking water. These industries are mostly confined to Katmandu valley. Some are established as beverage industries, other as mineral water industries and processed drinking water industries, and the others are named differently. They sell water in laminated plastic jar (20L) and in disposable plastic bottles in 1/2 to 2 liters (DFTQC, 2005/06).

This study wishes to access the quality of commercially packaged drinking water and mineral water available in Kathmandu valley of Nepal since such information is lacking in the Literature.

Materials and Methods

Forty eight samples of mineral water, natural spring water and processed or purified drinking water samples received in DFTQC and fifteen samples collected from different markets of Kathmandu valley (from 2006, August to 2007, August.)

were taken for analysis . Prior to analysis, samples were stored at 15 °C for chemical and radiological analysis and at 4 °C for the microbial analysis. Labels of samples were studied for its water source, treatment process, quality status, brand name and expiry dates on the basis of Codex guidelines (CAC, 1985).

Analytical Methods:

Chemical analysis

PH: Determined by using calibrated pH meter, *Hanna-2003,* at 25°C (Clesceri *et al.*, 2001). ***Total Dissolved Solid and Electrical Conductivity:*** Calibrated ***EC-TDS*** meter, *ELICO CM 183*, was used to measure the total dissolved solid by conductivity measurement at 25°C (Clesceri *et al.*, 2001). ***Ammonia:*** Spectrophotometric-Phenate method was used to measure ammonia quantitatively (Clesceri *et al.*, 2001) and Nessler's reagent was used for qualitative detection (Vogel, 1978). ***Sulphate:*** Total sulphate was changed into homogenous turbidity of $BaSO_4$ and concentration was measured by spectrometric method at 420nm using reagent blank (Clesceri *et al.*, 2001). ***Nitrite:*** Spectrophotometric method forming reddish-purple azo dye produced at pH 2-2.5 by coupling diazotized sulfanilamide with NED dihydrochloride with absorbance measurement at 543nm was used (Clesceri *et al.*, 2001). ***Chloride:*** Quantitative Mohr's method was used (AOAC, 2000). ***Residual Free Chlorine:*** The residual chlorine in sample liberates equivalent amount of free iodine from potassium iodide in at pH 3-4 of acetic acid and the liberated iodine was determined by titration with standard sodium thiosulphate solution (AOAC, 2000). Chlorex kit was used for qualitative test (DFTQC, 2005). ***Total hardness as $CaCO_3$:*** EDTA titration method at pH-10 using solochrome indicator was used (Clesceri *et al.*, 2001). ***Alkalinity as HCO_3:*** Total alkalinity was measured by titrating sample with 0.05M HCl using mix indicator with Bromocresol green and Methyl red (DFTQC, 2005). ***Iron:*** Phenanthroline method was used to determine total iron (Clesceri *et al.*, 2001). ***Arsenic:*** Spectrophotometric SDDC method was used for quantitative determination of arsenic at µg/l level (DFTQC, 2005). ***Calcium, Magnesium, Zinc, Copper, Lead, and Cadmium***: Sample was digested with nitric acid, neutralized and measured by using AAS, *HITACHI 1987* (Clesceri *et al.*, 2001)

Radiation Activity: Becquerel monitor *LB200, BERTHHOLD* was used to measure the radiation activity in Bq/l (DFTQC, 2005).

Microbial analysis

Total Mesophelic Count: The sealed water sample bottle was shaken vigorously. Serial decimal dilution were made using buffer peptone water. 1ml of different dilution of mineral water was plated with plate count agar and nutrient agar and incubated at 37°C for 48h and counted the colonies (DFTQC, 2005). ***Fecal Coliform and E. coli:*** Samples were analyzed using brilliant green bile broth (2%) tubes with Durham's tube inside and incubated at 37°C for 48 h and then counted the positive tubes. For faecal coliforms, from each positive tube, two loopfuls of inoculums was transferred to E.C broth tube, with Durham's tube inside and incubated at 44°C for 48 h and then counted positive tubes. The bacterial density was estimated from MPN Tables. For *E. coli*, inoculums from positive E.C broth tube were streaked on EMB plates and incubated at 37°C for 48 h and then proceeded in IMVIC test (DFTQC, 2005). ***Salmonella:*** 25 ml of sample was pre-enriched with 225 ml of buffered peptone water for 18-24 h and then enriched with tetrathionate broth or selenite cystine broth and then incubated at 44°C for 48 h. Inoculums from enriched broth were streaked in a Brilliant green phenol red agar or Bismuth sulphite agar and incubated at 37°C for 48 h. The plates were examined after 24 and 48 h for typical colonies of salmonella sps. The positive cultures were screened on TSI (Triple Sugar Iron) slant and confirmed by biochemical and serological test (DFTQC, 2005).

Statistical Analysis: Statistical data analysis was carried out as described by Gupta & Kapoor (2004).

Results and Discussion

Received and collected samples were analyzed within seven days of collection. Among the 65 samples analyzed, 43 samples were found within Nepal Standard for chemical quality (Table 1) and 38 samples were found within the Nepal standards for microbiological quality (Table 2). The chemical parameters which exceeded the Nepal Standard more frequently upon analysis of water samples were pH, ammonia, iron, residual chlorine zinc and nitrite (Table 1). Whereas the most frequent microbiological parameter that exceeded the Nepal standard was Total Mesophilic Count (Table 1). Eleven samples had lower pH value (<6.50) deviating towards acidic side. TDS of packaged water was not found exceeding 200 ppm.The hardness and alkalinity were 164 and 281ppm maximum respectively. Ammonia was detected in 3 samples and residual free chlorine was found fairly higher (>0.2 ppm) in 9 samples. Nitrite was detected in 7 samples and two exceeded the guideline value (<0.005ppm). The maximum amount of iron was 0.47ppm and most of the samples had fairly lower value of iron. Similarly zinc was found in 38 samples within the National standards (5.0 ppm), but had higher than WHO guideline value (0.5 ppm). Calcium, Magnesium, Chloride and Sulphate were less than 50 ppm in packaged drinking water. Copper, Lead and Cadmium were not detected up to 0.1ppm level. Arsenic was detected upto 20 ppb in nine samples. The amount of arsenic analyzed was less than the tolerance limit of Nepal Standard i.e. 50 ppb.

The radiation activity was detected in 41 samples with max. of 19Bq/l among 19 natural spring or mineral water and of 23Bq/L among 22 processed or purified drinking water. It needs to detect the radionuclides present in the water to judge safety (WHO, 2004). The radon-222 present virtually everywhere in the earth, the average concentration of radon is usually less than 0.4 Bq/l if source is public water supplies and about 20

Table-1: Chemical Quality and Radiation Activity of packaged drinking water available in Kathmandu Valley

Parameter	X_1/X_2	*Natural Spring /Mineral Water*						*Possessed or purified Water*				
		S_1/S_2	*Max. Value*	*Min. Value*	*Gross Mean*	*SD*	s_m	S_1/S_2	*Max. Value*	*Min. Value*	*Gross Mean*	*SD*	s_m
PH (25^0C)	6.5-8.0/ 6.5-8.5	28/3	7.9	5.5	7.0	0.61	0.22	37/8	7.9	5.5	6.8	0.53	0.2
TDS (25^0C)	<750/<10 ; >250*	28/0	166	4.7	50.7	44	16.3	37/0	165	4.6	48.9	49.9	16.3
Conductivity (μS)	NA	28/0	230	12.4	54.1	22.9	8.47	37/0	311	13.8	68.7	27.3	8.9
Ammonia (ppm)	0/NA	28/1	0	0	0	--	---	37/2	9.6	0	0.08	0.43	0.14
SO_4^-(ppm)	250/25	28/0	11.9	0.9	3.7	1.41	0.52	37/0	13.9	0.7	1.9	1.84	0.6
Cl^- (ppm)	<200/ <250	28/0	33.9	1.0	8.7	9.30	3.5	37/0	65.9	2	18.2	24.1	7.9
Nitrite (ppb)	<5/<5	28/1	24	0	0.29	1.1	0.4	37/2	4.0	0	0.01	0.01	0.01
Hardness as $CaCO_3$ (ppm)	<200/NA	28/0	150	2	20.4	27.6	10.2	37/0	164	2	42.3	44.9	14.7
Alkalinity as HCO_3 (ppm)	<600/NA	28/0	280	24.4	106	67.7	25.1	37/0	203	14	92.6	47.9	15.7
Free Res. Chlorine (ppm)	<0.2/<0.1	28/4	0.5	0	0.08	0.05	0.02	37/5	1.0	0	0.2	0.2	0.08
Iron (ppm)	<0.3/<0.3	28/0	0.17	0	0.1	0.05	0.02	37/4	0.47	0	0.11	0.1	0.03
Calcium (ppm)	<100/NA	28/0	8.6	0.22	2.1	3.07	1.4	37/0	9.6	0.22	2.5	2.5	0.83
Magnesium (ppm)	<75/NA	28/0	4.0	0.02	0.58	1.12	0.41	37/0	2.7	0.02	0.57	1.1	0.34
Zinc (ppm)	<5.0/<0.5	28/0	1.7	0	0.27	0.43	0.16	37/0	1.6	0	0.43	0.42	0.
Arsenic (ppb)	<50/<10	28/0	20	0	3.4	3.14	1.2	37/0	40	0	12.7	4.9	1.9
Copper (ppm)	<1.0/<1.0	28/0	0	0	ND	0	0	37/0	0	0	ND	0	0
Lead (ppb)	<50/<10	28/0	0	0	ND	0	0	37/0	0	0	ND	0	0
Cadmium (ppb)	<10/<3.0	28/0	0	0	ND	0	0	37/0	0	0	ND	0	0
Radiation Activity (Bq/L)	NA/ 20	28/0	19	0	7.5	7.5	2.8	37/0	23	0	3.92	5.7	1.9
Total (S_1/S_2)		**28/6**						**37/16**					

S_1=Total samples analyzed; S_2= Total samples substandard w.r.to Nepal Standard
s_m= Uncertainty of measurement about gross mean at 95% confidence limit.
ND = Not Detected by Analytical Technique
X_1= Nepal standard for drinking water; X_2= WHO Guideline for Drinking Water Quality
*=WHO Guide Line value for natural mineral water.

Bq /l for ground water source (IPCS, 2000; WHO, 2004). Control measure is to be taken if the radon concentration of drinking water for public supplies exceeds 100Bq/l.Screening level for drinking water are 0.5 Bq/l for gross alpha activity and 1.0 Bq/l for gross Beta activity (US NAS, 1999).There is evidence from both human and animal studies that radiation exposure at low to moderate doses may increases the long term incidence of cancer and the rate of genetic malfunction may be increased by radiation exposure (US NAS, 1999) .

Microbiological analysis showed that total mesophelic count (TMC) was above the National Standard in 29 samples (Table 2). It indicates higher susceptibility of contamination and poor sanitary condition of water sources and processing plants as well as longer age of the samples. Fecal coliform and E. coli were detected in 6 (9% of total) samples . Salmonella was not detected in any samples. Microbial quality of water may vary rapidly and widely with time interval and some enteric virus and protozoa are more resistant to disinfection and result of water quality testing for microbes at particular time may not assure the microbial safety of water (Dufour, 2003; IPCS, 2000).

After 30 days of storage , twenty four water samples became turbid and showed algal growth .The literature showed that bacterial growth increases rapidly after 30 days storage and become undrinkable after 270 day. High level of turbidity can protect microorganisms from the effect of disinfection. So it is essential to mention expiry date of less than one month in packaged drinking water (Dufour, 2003; IPCS, 2000).

Table-2: Microbiological Analysis of packaged or bottled drinking water available in Kathmandu Valley

Guideline Values	*Salmonela /25ml*		*E.Coli /100ml*		*Fecal Coliform /100ml*		*TMC /ml*	
X_1	-ve		-ve		-ve		25	
X_2	-ve		-ve		-ve		N.A.	
Water Type	+ve	-ve	+ve	-ve	+ve	-ve	$>X_1$	$<X_1$
Natural Spring or Mineral Water (S1/S2=28/13)	1	27	1	27	1	27	13	15
Processed or purified drinking water (S1/S2=37/16)	1	35	3	34	4	35	16	20

S_1 Total samples analyzed; S_2= Sub-standard samples w.r.to Nepal Standard
X_1= Nepal standard for drinking water; X_2= WHO Guideline for Drinking Water Quality
NA = Not Available.

The bottles and jars used for packaged water were recyclable plastic, poly ethylene terephthalate or PET (Dufour, 2003; IPCS, 2000). Bottles were generally found sterilized by hypochlorite solution or UV radiation before use. Even if the water itself pure, a plastic container upon repeated use may leak chemical such as phthalate or bisphenol-A which is suspected as carcinogen of endocrine system and may affect the order and taste acceptability of water (IPCS, 2000; WHO, 2004).

The analytical compositions giving characteristics to the product, information about source of water and expiry date shall be declared in the labeling (CAC, 1985) but details of that information were not mentioned in the labeling of 48 samples in this study. While sampling, water packaging container was found to be leaked in seven samples. Moreover, the brand names and treatment processes of packaged water were also exaggerated that pretends customers from actual quality and safety of drinking water.

Packaged, purified or processed, waters are for human consumption and may contain minerals, naturally occurring or intentionally added but shall not contain sugars, sweeteners, flavorings or other food stuff. Addition of minerals to water must comply with the provision of codex (CAC, 2001). The total dissolved solid analysis showed that mineral content in the both type of waters was found nearly same. The purified water is understood as de-mineralized or distilled water and contains less than 10 ppm of TDS. Natural spring or natural mineral water is defined by origin characterized by its content of certain mineral salts in a definite proportion and the presence of trace elements or other constituents with TDS 250 ppm within its protected perimeters (CAC, 1997). Mineral water samples had low mineral contents i.e. TDS lower than 250 ppm and purified drinking water had high mineral content i.e. TDS higher than 10 ppm (Table 1). Natural mineral or spring waters are collected from the spring, well or boring source strictly and only filtered and sterilized. The processed packaged water may be de-mineralized or with added mineral nutrients (Gleick, 2004; CAC, 1997). Natural mineral water is subjected only by the permitted physical treatment and packaged close to the point of emergence of the source with particular hygienic preconditions (CAC, 1997). Nepal has one standard for all types of packaged water (Nepal Gazette, 1998).

Conclusions

This study may reflect the alarming existing situation for the quality of packaged drinking water available in Kathmandu Valley. Packaging and labeling practices seem to be improved to the prescribed level. Following recommendations may be useful for insuring the quality of packaged drinking water, so as to protect the right of consumers, to have quality and safe drinking water.

- The safety policy for processed drinking water is to be enforced in practice. Protection of water resource, proper selection and operation of a series of treatment steps and management of packaging system, packaging material or container quality, and use of preservatives for long period storage is to be given high priority.
- The government acts and regulations for bottled or packaged water is to be revised for different types of packaged drinking water and is to be implemented for quality assurance.
- Inspections, testing and public awareness should be conducted as complementary activities. It is emphasized that sanitary inspection and water quality testing for source and end products is to be complementary and more frequent during licensing the packaged drinking water industries.
- Labeling of packaged drinking water is to be authentic and fully informative including all necessary ingredients that reflect true quality and safety for specified period of time.

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Coliform Load in Different Processing Steps and Sources of Post-contamination in Dairy Industries situated in *Kathmandu Valley*

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High microbial load associated with coliform bacteria in milk lower their keeping quality and might be source of potential risk for milk epidemics. For much of the twentieth century, coliform bacteria have been used as indicators of possible post-processing contamination in milk. Coliform count at different processing levels viz., raw milk before heat treatment, just after pasteurization, storage tank, overhead tank and packaged milk of four dairy plants situated in Kathmandu Valley were investigated. Results indicated that the coliform count of raw milk of all dairies was found high with average of 5.84 $\log_{10}$ 6.95 $\log_{10}$ 6.50 $\log_{10}$ and 6.41 $\log_{10}$ cfu per ml. Milk after pasteurization showed nil coliform in three dairies while in further processing steps their number increased and in final stage of processing reaching into 1.75 $\log_{10}$, 1.00 $\log_{10}$, 1.42 $\log_{10}$ and 2.07 $\log_{10}$ cfu per ml. The possible sources of post pasteurization contamination were also investigated and the results showed that air, water, storage tanks and packaging plastic films were the major sources of post processing contamination to the processed milk.

Keywords: Coliform, Raw milk, Packaged milk, Air, Water, Plastic.

Introduction

Milk is considered to be the perfect food and is especially valued for the manufacturing of most dairy products because of its high nutrient content (Charley & Weaver, 1998). However, milk is also an excellent growth medium for a variety of microorganisms (Cappuccino & Sherman, 2003; Fox & Cameron, 1982). Thus efficient heat treatment of milk is essential to eliminate any potential pathogenic and spoilage bacteria that may be present (Corash, 1951; Dubis, 1998).

The sources of infection or contamination of milk may be the dairy animals, human handlers or the environment such as contaminated vessels, polluted water, flies, dust etc (Adam & Moss, 2002). The spoilage of milk by microbes results in the alteration of milk components, colour, flavour, gas and production of toxin finally leading to economic losses (Jensen & Vorup, 1989).

The coliform bacteria belongs to family *Enterobacteriaceae* which comprises all aerobic and facultative anaerobic, gram-negative, non-spore forming rods capable of fermenting lactose with the production of acid and gas at 32°C within 48 h. Typically, these organisms are classified in the genera *Escherichia*, *Enterobacter* (formerly *Aerobacter*) and *Klebsiella* (Nelson, 1985) and number of species belonging to this group may be 20 or more (Hitchins *et al.*, 1992). While the general source of these organisms is commonly accepted to be the intestinal tract of warm blooded animals, it is emphasized that both fecal and non-fecal origin are members of this group. The existence of any of these types in dairy product is suggestive of unsanitary conditions or practices during production, processing or storage. Since coliform display little heat resistance, their count can be useful when testing for post-processing contamination of processed foods (Dogan-Halkman *et al.*, 2003).

Cleaning in place (CIP) is the most commonly used practice for cleaning and sanitizing in large milk processing plants. However, even after the use of acceptable cleaning processes, soil residues and microorganisms remain on the contact surfaces. There is also possibility of air as a vehicle for allowing pathogenic organisms to enter in dairy products (FDA, 1988; Kang & Frank, 1989). Some potential sources of aerosolized bacteria are floors, drains, condensate, personnel, outdoor air, and air conditioning systems (Kang & Frank, 1990).

The microbial problems can be minimized by the heat treatment of milk such as pasteurization, sterilization etc. Default process or post process contamination makes milk unsafe for human consumption. This has made coliform enumeration in milk more important for tracing out the probable cause or point of contamination. This process may help in improving the processing technique in future.

Poor cleaning and inadequate microbiocidal treatment of equipment with which the pasteurized milk comes in contact undoubtedly are the most important sources of contamination with coliform bacteria. Unsanitary practices are additional sources of contamination (Nelson, 1985).

The objective of this study is to evaluate the microbial quality of milk at various flow levels viz., raw milk before heat treatment, just after pasteurization, storage tank, overhead tank, packaged milk with respect to coliform bacteria and identification of sources of post processing contamination.

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Materials and methods

The samples required for this study were collected from the four dairies, two having continuous processing facilities through plate heat exchanger (PHE) and the rest having batch. As far as possible, the same lot of milk was used for analysis at different stages. The samples of pasteurized packaged milk were taken directly while milk from other different stages of processing was taken in the sterile 50 ml bottle in properly labeled condition and transported within two hrs in the ice-box maintaining temperature at about 4°C.

Violet Red Bile Agar (VRBA) media was used to determine the coliform count at 30 ± 1°C for 24 ± 2 h and the purplish red colonies having diameter of at least 0.5 mm diameter surrounded by a reddish zone of precipitated bile was counted as coliform. Liquid VRBA medium at 44-46°C was poured in the petridish containing 1 mL of diluted sample in the centre of the plate and about 15 ml medium was placed, mixed well and after completely solidification another 5 mL molten medium was overlaid as described in NDDB (2001). The average coliform count of all milk samples were expressed as $\log_{10}$ cfu/ml. When the count was zero for coliform count, a value of 1 per ml was assigned to the sample; hence the $\log_{10}$ value was calculated as zero for statistical analysis.

The coliform in air was measured by settling plate method. In this method the cover of the petri dishes containing VRBA medium were removed and the agar surface was exposed to the air for two hrs in different areas of the milk processing plant. The microorganisms were collected or settled directly on the solid surface of agar medium and colonies were developed on the medium. Then after two hrs, petri plates were covered and incubated at 30 ± 1°C for 24 ± 2 h. Each colony represents a particle carrying microorganism.

Results and Discussion

Coliform load in raw milk

A total of 40 samples of raw milk before heat treatment from four different dairies were examined for coliform bacteria. It was observed that coliform count was ranging from maximum 7.83 $\log_{10}$ to minimum 5.50 $\log_{10}$ cfu/ml, with a mean of 5.84 $\log_{10}$, 6.95 $\log_{10}$, 6.50 $\log_{10}$ and 6.41 $\log_{10}$ cfu/ml for four dairies (A, B, C and D) respectively (Table 1).

Table 1: Coliform load in raw milk from different dairies situated in *Kathmandu* Valley

Dairy	*Mean*
A	5.84^{a} (6.74-5.50)
B	6.95^{b} (7.83-6.62)
C	6.50^{c} (6.99-5.87)
D	6.41^{c} (6.99-5.65)

Values are expressed as $\log_{10}$ cfu/ml.
Figures in parentheses denote the range of data of 10 observations.
Different letters in superscripts indicate that the values are significantly ($p<0.05$) different

The statistical analysis showed that mean Coliform load in raw milk differs significantly at $p<0.05$ level. High Coliform count in the raw milk reflects that the hygiene and sanitation is not satisfactory at the milk production, collection and transportation stages.

In view of quality improvement, there is no mandatory standard for coliform count in raw milk in Nepal. However, the mean coliform counts observed in this study were higher than those reported by Hogan *et al.* (1988); Jayarao & Wang (1999), which were reported to be 2.0 $\log_{10}$ cfu/ml and 3.4 $\log_{10}$ cfu/ml respectively. Bray & Shearer (1996) have suggested guidelines to interpret coliform counts in raw milk and based on these guidelines, a count greater than 3.0 $\log_{10}$ cfu/ml suggests problems associated with cleaning of the milking system or improper disinfection of teats before milking.

Jayarao *et al.* (1999) have suggested that the probable route of transmission of fecal origin subtypes of *E. coli* could be through temporary habitation of the streak canal between milking, during milking, would gain access into raw milk. Coliform bacteria can also gain access directly into bulk tank milk when cows with sub clinical Coliform mastitis are milked and have been shown to increase the coliform counts of bulk collection (Bramley & Mckinnon, 1990).

Coliform load in milk from just after pasteurization (JAP)

The coliform count was found maximum 1.54 $\log_{10}$ cfu/mL to zero cfu/mL, with mean count nil for dairies A, B, and C and 0.57 $\log_{10}$ cfu/ml for dairy D (Table 2).

Table 2: Coliform load of milk JAP of different dairies situated in *Kathmandu* Valley

Dairy	*Mean*
A	0.00^{a} (0)
B	0.00^{a} (0)
C	0.00^{a} (0)
D	0.57^{b} (1.54-0)

Values are expressed as $\log_{10}$ cfu/ml.
Figures in parentheses denote the range of data of 10 observations.
Different letters in superscripts indicate that the values are significantly ($p<0.05$) different

Three different dairies A, B and C did not show coliform while dairy D had shown in some cases. Various research workers agreed that the pasteurization does not kill all the microorganisms present in raw milk, especially thermoduric type. The presence of coliform in case of dairy D might be due to presence of some coliform which might survive pasteurization temperature for few seconds. Other probable cause of coliform count in JAP milk in D type dairy may be due to insufficient heat treatment during pasteurization.

Coliform load in milk from pasteurized milk storage tank (PMST)

The coliform count was found maximum of 2.07 $\log_{10}$ cfu/mL to zero cfu/mL, with mean of 1.00 $\log_{10}$, 0.13 $\log_{10}$, 0.63 $\log_{10}$ and 0.88 $\log_{10}$ cfu/ml for dairy A, B, C, and D respectively (Table 3).

The Coliform count increased significantly than the previous step, i.e., JAP. The increase in count could be attributed to the post pasteurization contamination in all cases. It was found that the dairies having higher number of coliform in air and in water showed higher number of coliform in milk from PMST. The coliform count at this stage may be due to improper cleaning procedure. The swab test of storage tank of dairy A was found having maximum contamination compare to other dairies.

Table 3: Coliform load in milk from PMST of different dairies situated in *Kathmandu* Valley

Dairy	*Mean*
A	1.00^{a} (2.07-0)
B	0.13^{b} (0.69-0)
C	0.63^{ab} (1.81-0)
D	0.88^{a} (1.99-0)

Values are expressed as $\log_{10}$ cfu/ml.
Figures in parentheses denote the range of data of 10 observations.
Different letters in superscripts indicate that the values are significantly ($p<0.05$) different

Milk from all dairies showed post pasteurization contamination. The control measures applied by all dairies were same. Improper cleaning of milk storage tank and the possible contamination during sampling of milk may be the reason for high coliform count.

Coliform load in milk from overhead tank (OHT)

The coliform count in milk from OHT was observed maximum 2.30 log10 cfu/mL to zero cfu/mL, with mean of 1.61 $\log_{10}$, nil, 1.03 $\log_{10}$ and 1.41 $\log_{10}$ cfu/mL for dairy A, B, C and D respectively (Table 4).

Table 4: Coliform load in milk from OHT of different dairies situated in *Kathmandu* Valley

Dairy	*Mean*
A	1.61^{a} (2.30-1.00)
B	0.00^{c} (0)
C	1.03^{b} (1.81-0)
D	1.41^{ab} (2.22-0.84)

Values are expressed as $\log_{10}$ cfu/ml.
Figures in parentheses denote the range of data of 10 observations.
Different letters in superscripts indicate that the values are significantly ($p<0.05$) different

The total number of coliform increased significantly in all except in the case of dairy B and there was significant difference ($p<0.05$) in the procedure applied by all the four dairies. The increase in coliform load than the previous stage was due to the post processing contamination via air, water and improperly cleaned and sanitized overhead milk storage tank. Dairy B had better procedure of sanitization than the rest of the dairies by circulating hot water at 80°C for few minutes to pasteurized milk storage tanks, overhead tanks and pipelines.

Coliform load in packaged milk

The Coliform count of packaged milk ranged from maximum of 3.04 $\log_{10}$ cfu/mL to zero cfu/mL, with mean of 1.75 $\log_{10}$, nil, 1.42 $\log_{10}$ and 2.07 $\log_{10}$ cfu/mL for A, B, C and D dairy respectively (Table 5).

Table 5: Coliform load in packaged milk of different dairies situated in *Kathmandu* Valley

Dairy	*Mean*
A	1.75^{ab} (2.12-1.25)
B	0.00^{c} (0)
C	1.42^{b} (1.9-0.95)
D	2.07^{a} (3.04-1.32)

Values are expressed as $\log_{10}$ cfu/ml.
Figures in parentheses denote the range of data of 10 observations.
Different letters in superscripts indicate that the values are significantly ($p<0.05$) different

Nepal Gazette (2001) has set up maximum permissible limits for coliform in pasteurized milk is zero per mL. The coliform count in pasteurized milk at different process stages of dairy B was observed zero except at PMST. The presence of coliform in pasteurized milk at PMST may be due to contamination during plating process. The absence of coliform count at different process stages of dairy B indicates proper milk pasteurization process and good sanitation during all steps of milk processing. In case of other dairies A, C and D the coliform count in pasteurized milk at different stages of processing showed very high count. This indicates improper pasteurization and post pasteurization contamination at different stages through water, air, handler, plastic film etc.

The cleaning and sanitizing procedure of all equipment and materials which comes in contact with pasteurized milk is very important. The destruction of coliform from plastic film by UV treatment is an important step which only dairy B had that facility. It might be due to this reason dairy B had no contamination through packaging film.

Coliform load in air, water and plastic film

The air in all the dairies was heavily contaminated by coliform (Fig. 1). The mean and range of coliform count of dairy A, B, C and D were found 10.6 (25-2), 2.1 (6-0), 3.8 (8-0) and 6.0 (15-0) cfu respectively. Since this technique does not record the

volume of air actually sampled, it gives only a rough estimate. However, it does give information about the kind and number of microorganisms in a particular area (Powar & Daginawala, 2003). The number of microorganisms is influenced by size of particles and by the speed and direction of air flow (Jay, 2005).

Canon (1966) studied the presence of microorganisms in air in ten different dairy plants and coliform were found to be of 1% to the other bacteria and molds. According to the Shah *et al.* (1996) principal sources for the presence of air-borne microorganisms in the dairy factories are human, floor drains, ventilation systems, unsanitary refuse, raw materials, incoming goods, milk spills and outside dust.

The mean and range of coliform count in water of dairy A, B, C and D was found 10.1 (40-2), 13.0 (29-2), 6.7 (12-2) and 340.1 (1120-12) cfu/mL respectively. The water from all dairies was heavily contaminated with coliform. Although dairy B has UV rays treatment for water it treated only a portion of water which was not enough. Similarly dairy D has *euroguard* for water treatment while rest dairies were using untreated water directly for washing and cleaning. The use of untreated water directly to processed milk increases the coliform load. However, the water treatment facility in all the dairies seemed unsatisfactory and needs prompt action towards improvement.

Ten different samples from each dairy was analysed for coliform in plastic film and the mean and range of coliform count of dairy A, B, C and D was found 1.5 (5-0), 0 (0), 2.1 (5-0) and 13 (35-3) cfu/4cm^2 respectively.

The coliform bacterial load in plastic film is directly related with the quality of air, dust/smokes present, storage condition and time of exposure of film from rolls. The plastic films used by all the dairies were heavily contaminated by coliform bacteria (Fig. 2) except dairy B, which was equipped with UV treatment facilities.

Coliform load in cleaning and sanitization process

Pasteurized milk storage tank and packing machine could be other post contamination sources and they were investigated during study. Ten different samples from each dairy was analysed and the mean and range of coliform count of dairy A, B, C and D was found 0.7 (3-0), 0.4 (2-0), 0.4 (2-0) and 0.4 (2-0) cfu/4cm^2 respectively in the storage tank. Likewise, the mean and range of coliform count of dairy A, B, C and D was found to be 5.1 (18-0), 0 (0), 33.7 (109-0) and 26.3 (119-0) cfu/4cm^2 respectively in the packing machine.

The coliform load was observed high in PMST in all dairies (Fig. 3). The high coliform count is due to improper cleaning and sanitation of milk storage tank. The use of unclean water and air significantly contribute to high microbial load. Likewise, packaging machine of A, C and D were found heavily contaminated with coliform. It might be due to the improper cleaning and sanitation procedure.

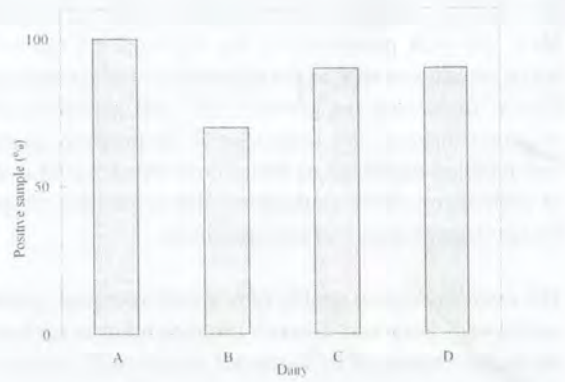

Fig. 1: Coliform load in surrounding air from different dairies situated in *Kathmandu* Valley

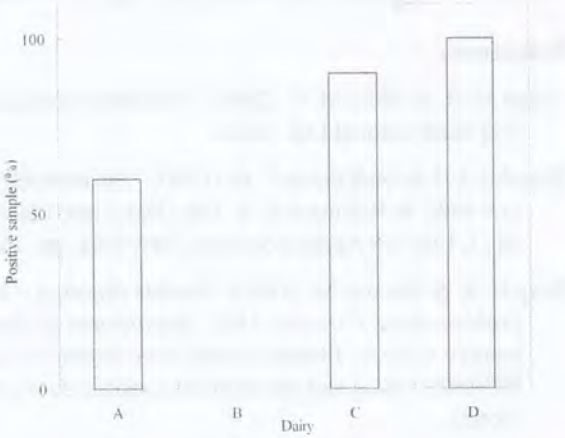

Fig. 2: Coliform load in packaging plastic film from different dairies situated in *Kathmandu* Valley

Fig. 3: Coliform load in PMST and packaging machine from different dairies situated in *Kathmandu* Valley

Conclusions

Milk and milk products are the vehicle for food and water borne diseases as well as the major source of zoonotic disease. Hence, to prevent the public from such communicable and zoonotic disease, the milk should be properly pasteurized and handled carefully to avoid contamination at every step of processing, from pathogens and organisms responsible for spoilage of milk and milk products.

The microbiological quality of raw milk received by all dairies seems very poor and it needs improvement at earliest. Only the pasteurization of milk can not ensure milk safe for human consumption unless all the processes after pasteurization is carried over properly and to avoid post pasteurization contamination. Besides proper pasteurization of milk proper cleaning and sanitization of equipments, good water and air is required to produce coliform and pathogen free milk.

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Bacteriological Risk Analysis of Rural Water Supply Schemes in Western Development Region of Nepal

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The study assessed the bacteriological risk of rural water supply scheme from six different districts in western development region of Nepal. It covered 68 schemes focusing on the rural drinking water supply of Gorkha, Kaski, Lamjung, Nawalparasi, Syangja and Tanahu districts. Water Samples were collected from source, reservoir tank, tap stand and household level. The water when travels from source to distribution line, and to household, density of the Fecal Coliform was found to increase. The water quality in terms of bacteriological parameter such as Fecal Coliform showed variation in the different points of water distribution. The study revealed that water supplied in rural drinking water schemes do not meet the WHO standard and population in western development region in Nepal were under high risk of fecal contamination.

Keywords: Bacteriological risk analysis, Rural water supply schemes, Western Development Region of Nepal.

Introduction

Nepal is predominantly a rural country with nearly 85 percent population living in rural area. Sixty six percent of the rural population has an access to piped water, and others are dependent on locally available water sources such as spring, stream and tube well for drinking and household uses. The per capita water consumption in the rural area is 45 liter as compared to 60 liter in the urban area. (ADB, 2006)

One of the major problems of the rural areas is poverty associated with an environmental sanitation. Out of the total consumption of water in the domestic sector, only 2% is used for drinking purpose, rest is used for cleaning and hygiene (ADB, 1985). There is an acute shortage of safe drinking water. Some of the advantages of having water available at village taps are saving of time (opportunity to engage in other income generating activities especially to women) and its positive impact on the users on health, domestic hygiene (toilet facilities), environmental hygiene (drainage pattern) and socio economic gain (water haulting time) (Pyakurel, 1994). A community water supply can be of great benefit to the health and the well being of people to whom it serves, it can be, on the other hand, cause of disease and a serious threat to the public health if the delivered water is contaminated. Recurrent outbreaks of water borne epidemics and increasing number of patients and high infant mortality rate due to water related diseases indicates that only supplying of drinking water is not sufficient to improve public health status unless supplementary effort is made both on quantity as well as quality aspect of water supply and sanitation (Sharma, 1993).

Infectious disease caused by bacteria, viruses and protozoa or by other helminthes parasites are the most common and wide spread health risk associated with drinking water. The diseases are transmitted particularly through human and animal excrete, particularly feaces (WHO & UNICEF, 2004).

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Many studies have related to very high content of coliform bacteria in portable water in both Terai and hilly regions of Nepal. In rural area 16.7% to 33.3% and in urban area 70 to 100% water samples were found to be contaminated with coliform bacteria. Almost all piped water supply are contaminated during rainy/summer season, and water quality is far from satisfactory. Serious efforts are needed to reduce bacterial contamination in potable water (Sharma, 1994).

Nation faces a worsening series of local and regional water quantity and quality problems, largely as a result of poor water allocation, wasteful use of the resource, and lack of adequate management action. Water resources constraints and water degradation are weakening one of the resource bases on which human society is built. This study aims to assess bacteriological risk associated with the types of sources, number of taps and population under risk in western development region of Nepal.

Materials and Methods

The study was conducted in six districts of Western Development Region of Nepal; *Gorkha, Kaski, Lamjung, Nawalparasi, Syangja* and *Tanahu*. This region covers an area of 29398 Sq. Km and lies between $27^0 30'$ and $29^0 20'$ latitude, and $82^0 49'$ and $85^0 15'$ longitude. The total population is about 4,571,013 (CBS, 2001). The study area covered representations from all the three major ecological regions of Nepal namely; Terai, Hill and Mountain. The study covered a total of 68 rural drinking water supply schemes of six districts.

Tools and Technique of Data Collection

Primary data used in the study was collected by using questionnaire method. A set comprising two questionnaires were used so as to gather information on the technical knowledge, attitude and practice of the beneficiary population.

- *Community Interview Questionnaire*: This was used to gather information on the community participation

on operation and maintenance of the systems. Interview with focus groups such as Water User Committee (WUC) was held to gather information on technical details of the schemes.

- *Household Interview Questionnaire*: This was used to assess the knowledge and attitude of the respondents in personal, domestic and environmental health and community participation. Same questionnaire was used for two different target groups on the basis of different living standards; one elite and another underprivileged household.

Collection of Water Samples

Water Samples were collected from source, reservoir tank, tap and household level, to investigate the risk level in the current rural drinking water supply schemes. Total water samples collected were 497.

Bacteriological Analysis of Water

Bacteriological analysis of water samples was carried out using Membrane Filter Method (MFM) to detect the presence and its risk level of Fecal Coliform to human health in study area. Known volumes of diluted or undiluted water samples were filtered through 0.45-µm pore filters and incubated these filters directly on Endo medium. Colonies of Fecal Coliform were with a characteristic metallic sheen (Shakya, 1995). Risk measurement was carried out using WHO Risk measurement grade assigned as Bacteriological Risk Grade (BRG) that is presented in Table 1 (WHO, 1993).

Table 1: WHO Risk measurement and Bacteriological Risk Grade

Fecal Coliform Count/100ml	*Bacteriological Risk Grade (BRG)*	*Risk Grade*
0	1	No Risk
1-10	2	Low Risk
11-100	3	High Risk
101-1,000	4	Very High Risk

Representative Bacteriological Risk Value (RBRV)

Representative Bacteriological Risk Value (RBRV) is defined as the value which is total sum of the product of total population served for a scheme and its BRG to the total population served for the District.

$$RBRV = \frac{\text{Population Served for scheme}_1 \times BRG_1 + \text{Population Served for scheme}_2 \times BRG_2 + \text{Population Served for scheme}_3 \times BRG_3 + \text{and so on}}{\text{Total Population Served by schemes in a district}}$$

Results and Discussion

Bacteriological Water Quality at different points of water distribution

Bacteriological water quality analysis from six districts showed different levels of risk to human health. Fecal Coliform showed variation on the values in the four different sampling points of water distribution system (Table 2). The trend of Fecal Coliform from water source to household of six districts showed the similar increasing trend.

Table 2: Coliform load at different points of water distribution from six districts

District	*Total No of Schemes*	*Source*			*Reservoir Tank*			*Tap*			*Household*			*Total Num*
		Num	*Max*	*Min*	*Num*	*Max*	*Min*	*Num*	*Max*	*Min*	*Num*	*Max*	*Min*	
Gorkha	10	20	120	0	17	84	0	20	138	0	20	206	61	**77**
Kaski	10	17	33	0	23	73	0	20	72	2	20	209	0	**80**
Lamjung	3	3	54	0	3	91	51	6	97	66	6	113	78	**18**
Nawalparasi	20	27	82	0	32	188	3	40	130	10	40	256	0	**139**
Syangja	9	12	35	0	13	124	0	18	123	13	18	218	8	**61**
Tanahu	16	30	32	0	28	181	7	32	249	0	32	498	18	**122**
Average			**59**	**0**		**123**	**10**		**135**	**15**		**250**	**27**	
Total	**68**	**109**			**116**			**136**			**136**			**497**

*Num: Total Number of water samples collected
Max: Maximum value of fecal coliform count/100ml
Min: Minimum Value of fecal coliform count/100ml

Representative Bacteriological Risk Value (RBRV) of six districts at different points of water distribution

Average value of RBRVs from six districts showed that the risk level increases from water source to household level in all Districts. The average value at source was 1.95 that increase in reservoir tank to 2.7, 3 in tap and 3.32 in household (Table 3). RBRV showed that *Lamjung* was at highest level of contamination at source, reservoir tank and household where as *Syangja* was highly contaminated at tap. The average value of RBRV clearly shows that there was an increase in the slope of fecal coliform value from source to household. Reason in variation in RBRV of different districts may be due to different level of awareness on health and sanitation, geological setting around the drinking water scheme and community participation.

Table 3: RBRV of Drinking water from water source to household level of six districts

District	*Source*	*Reservoir Tank*	*Tap*	*Household*
Gorkha	1.9	2.8	3	3.6
Kaski	1.8	2.4	3	3.1
Lamjung	2.4	3	3	3.7
Nawalparasi	2.1	2.6	2.9	3
Syangja	1.6	2.5	3.1	3.2
Tanahu	1.9	2.9	3	3.3
Average RBRV	**1.95**	**2.7**	**3**	**3.32**

Bacteriological Risk Grade (BRG) at different points of water distribution

There were total 68 schemes with three types of sources namely ground water, spring and stream. Out of three types of source examined, stream was more likely to be contaminated than spring source (Table 4). More risk of contamination of stream source may be due to pollution like human settlement and animal farming in upstream, domestic and agricultural runoff, and landslide due to deforestation. Contamination of water at source point may be due to lack of protection against surface contamination, poor drainage system, unsanitary environment at surrounding of water source, lack of protection against silt and open defecation. In some case, nonfunctional masonry or concrete wall or spring box, unscreened intake and unprotected fencing around and other external factor like heavy rainfall during monsoon period were the reasons of contamination.

Table 4: Bacteriological Risk Grade at different points of water distribution

Water Source	*Bacteriological Risk Grade*				
	No Risk	*Low Risk*	*High Risk*	*Very High Risk*	*Grand Total*
Ground water	1	-	-	-	1
Spring water	16	11	10	-	37
Stream water	7	14	8	1	30
Grand Total	**24**	**25**	**18**	**1**	**68**

Bacteriological Risk Grade (BRG) of Tap water

From every scheme, water samples were collected which was 10% of sampling size. (136 water samples from 1337 taps). The study showed that 85% of total taps were at high risk, 8% at very high risk and 5% were at low risk (Fig 1). Increase in fecal contamination in taps may be due to leakage in main pipes and breakage or erosion in tap stands and poor drainage system of waste water around parish like open hole collection or collection in farmland, exposure of pipes near tap stand, unhygienic environment around parish like open defecation of animal and human, and open solid waste disposal and poor sewage management.

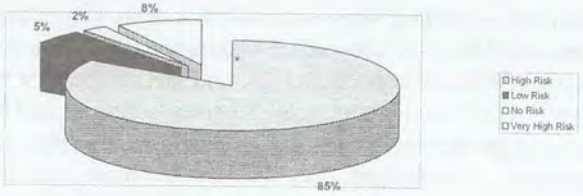

Fig. 1: Risk level of Water from Tap from six districts

Conclusions

The study instigated that the level of fecal coliform presence in water supplies in the rural drinking water schemes were mostly above the WHO standard. The average fecal coliform was found to be lowest in the source but the level of risk was very high in household level. Maximum populations were under high risk of fecal contamination. The main reason of such risk may be due to the lack of attention on health and hygiene. It is recommended to follow regular maintenance to control pollution and water availability, proper sanitation and hygienic practices. People should be educated on appropriate use of water, preventive measures and pace of technology evolution. Regular monitoring of rural water supply from concerned local authority is to be followed.

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Assessment of Microbiological Quality in Milk Chain of Dairy Development Corporation, *Balaju*

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Microbiological quality of milk in the entire milk chain of Dairy Development Corporation, Balaju, Kathmandu was studied to assess the quality of milk. Samples were drawn from the different stages of milk chain and were analyzed for the enumeration of total plate count, Gram negative count, Coliform and yeast mold count. The study was carried out in summer and winter season. Microbial counts were found to be high at farm level to just before pasteurization stage. Pasteurization step significantly decreased the microbial load. Milk after packaging showed a sudden increase in microbial load. Major Microfloras associated in raw and pasteurized milk were found to be Coliform, Micrococcus, and Corynebacterium. Hazard Analysis Critical Control Point (HACCP) system is to be implemented effectively in milk chain to improve the existing microbiological quality of milk

Keywords: Milk, HACCP, Pasteurization, Hygiene, Microorganisms

Introduction

Milk as it contains most of the proximate principles of a well balanced diet, is almost a complete and ideal food for human body (De, 1980). However, in spite of being milk as the best, ideal and a complete food for all age groups, it can also serve as a potential vehicle for transmission of some diseases under certain circumstances. Moreover, by virtue of possessing almost all the essential nutritional factors, milk can also serve as an excellent culture and protective medium for certain microorganisms which may include potential pathogens capable of causing serious health problems to the consumers (Bryan, 1992).

The milk, when secreted in a healthy udder is almost sterile. The contamination of milk with spoilage and disease producing microorganism may therefore occur at any stage of production, collection, processing, marketing and utilization (Pelczynska & Ezyaztof, 1995). Microbiological quality of the milk needs to be rigorously controlled with regard to both number and type of micro flora present (Adesiyun *et al.*, 1995).

Milk-borne diseases cause considerable morbidity and mortality throughout the world even though the principles for controlling most of these diseases are well established (Hunter, 1991). In Nepal, consumers are less conscious about the quality of food and people have very little knowledge of food hygiene. Microbiological quality of milk is studied in traditional ways. Since it is not possible to produce good quality market milk from bad quality raw milk, there is a need of monitoring of the bacteriological and hygienic quality of milk. It is necessary to find ways and means to improve it right from the onset of milk collection in the villages. Enhancing qualitative production needs the identification, assessment and control of the hazard quality, but it may fail to determine the point where hazard has occurred. Therefore, it cannot specify the criteria to ensure control; it neither takes any corrective action nor verifies the planned systems which in the milk chain (Easter *et al.*, 1994).

Considering these defects of the prevailing method, a new model system can be applied to achieve a good quality product which shares the quality assurance responsibility with all employees in a processing operation. This study assessed microbial hazards associated with the various stages in milk chain of Dairy Development Corporation, *Balaju*.

Materials and Methods

The study includes the enumeration of predominant bacteria present in milk; isolation and identification of the isolates. The work has been carried out in two seasons in a year i.e. summer and winter. The work was completed within January 1995 to March 1996.

Sample collection

A total of 282 samples were collected for microbiological examinations from different stages of milk chain. Three Chilling centers of Dairy Development Corporation (DDC) located at *Banepa*, *Panauti* and *Panchkhal*, and their respective farm levels were chosen for the study. Samples were collected from every steps starting from farmers' level to consumers' level following a method prescribed by Luck & Lategan (1984). Samples were collected from Farmer (three from each chilling centre); Milk producers Association (MPA); Chilling vat; Tanker at chilling centre; Tanker after reaching at Kathmandu Milk Supply Scheme (KMSS), *Balaju*; Storage tank; Before Pasteurization stage; After pasteurization; Homogenization; Before packaging ; After packaging (UV treated and untreated) and Consumer level. Besides these, water samples and milk Can rinsed water sample were collected from each chilling centre; Boiled water, Feed water, Tap water and package paper

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washed samples were taken from KMSS at *Balaju*. Can and package paper were rinsed by sterilized quarter strength solution of Ringer. All these samples were collected in a 200 ml sterilized glass bottle.

Enumeration of microorganisms

The enumerations of the sample were done for total viable aerobic microorganisms, Coliform bacteria, faecal Coliform and yeast & mold by serial dilution method (Benson, 1997). All the works except identification were done in the KMSS Microbiology laboratory at *Balaju*, while identification of isolates was done at the Central Food Laboratory, Department of Food Technology and Quality Control, *Kathmandu*, Nepal.

Results and Discussion

The result of microbilogical analysis of milk at various sampling points is summarized in Table 1.

Table 1: Microbial count of milk in summer and winter season (values in cfu/ml)

Location and samples	*Total Plate count*		*Gm-ve Bacteria*		*Coliform count*		*Faecal Coliform*		*Yeast and Mold*	
	Summer	*Winter*	*Summer*	*Winter*	*Summer*	*Winter*	*Summer*	*Winter*	*Summer*	*Winter*
Morning sample (M)	2.8×10^{7}	8.6×10^{6}	1.3×10^{5}	4.4×10^{4}	1.6×10^{3}	8.7×10^{3}	4.1×10^{3}	2.1×10^{2}	5.8×10^{3}	1.7×10^{3}
Evening sample (E)	4.5×10^{7}	1.6×10^{7}	7.5×10^{5}	1.2×10^{5}	7.0×10^{4}	3.2×10^{4}	2.9×10^{4}	2.7×10^{3}	4.4×10^{4}	3.2×10^{3}
Milk producer's Association (MPA)	7.9×10^{9}	1.1×10^{8}	3.3×10^{7}	5.5×10^{5}	2.9×10^{6}	1.5×10^{5}	9.1×10^{4}	2.2×10^{4}	8.2×10^{4}	3.9×10^{3}
Chilling vat (V)	1.1×10^{10}	1.3×10^{8}	3.5×10^{7}	8.2×10^{5}	3.1×10^{6}	1.6×10^{5}	1.0×10^{5}	2.4×10^{4}	9.0×10^{4}	4.4×10^{3}
Tanker before transportation (T)	1.3×10^{10}	1.6×10^{8}	4.0×10^{7}	9.2×10^{5}	4.1×10^{6}	1.8×10^{5}	1.3×10^{5}	2.4×10^{4}	9.7×10^{4}	4.9×10^{3}
Tanker after transportation (F_0)	1.4×10^{10}	2.0×10^{8}	4.1×10^{7}	1.2×10^{6}	4.4×10^{6}	3.2×10^{5}	1.4×10^{5}	2.8×10^{4}	9.7×10^{4}	5.0×10^{3}
Raw milk storage tank (F_1)	1.5×10^{10}	3.1×10^{8}	5.3×10^{7}	1.7×10^{6}	5.1×10^{6}	4.3×10^{5}	1.6×10^{5}	4.1×10^{4}	1.1×10^{5}	2.9×10^{4}
Before Pasteurization (F_2)	1.5×10^{10}	3.5×10^{8}	5.4×10^{7}	2.5×10^{6}	5.5×10^{6}	5.0×10^{5}	1.8×10^{5}	4.1×10^{4}	1.3×10^{5}	3.8×10^{4}
After Pasteurization (F_3)	8.1×10^{6}	1.8×10^{5}	5.9×10^{2}	14	70	0	10	0	2	5
Homonization (F_4)	1.1×10^{6}	1.2×10^{5}	1.3×10^{2}	1	40	0	7	0	3	1
Before packaging (F_5)	1.0×10^{6}	1.2×10^{5}	1.0×10^{2}	1	42	0	7	0	3	1
After packaging (F_6)	6.5×10^{6}	2.0×10^{5}	3.3×10^{2}	16	67	9	12	8	6	2
Cold storage (F_7)	1.6×10^{6}	2.2×10^{5}	3.3×10^{2}	15	81	14	19	7	10	2
Consumer (C)	5.7×10^{6}	2.3×10^{5}	3.8×10^{2}	16	1.45×10^{2}	13	47	5	30	2
Milk Can rinsed (CW)	8.32×10^{3}	6.7×10^{3}	4.7×10^{2}	4.0×10^{2}	1.1×10^{2}	65	38	2	22	2
Water at chilling center (WC)	1.3×10^{5}	1.0×10^{5}	1.0×10^{4}	1.7×10^{3}	1.7×10^{3}	1.8×10^{2}	3.8×10^{2}	56	3.2×10^{2}	30
Boiling water (BW)	2	5	0	0	0	0	0	0	0	0
Feed water (FW)	59	70	2	2	0	0	0	0	0	0
Tap water (TW)	87	56	5	5	4	4	1	1	0	0
Package paper (P)	74	56	4	5	2	2	1	2	0	1
Air (A)	215	86	36	5	10	4	4	1	64	5
Skim milk (F_6')	1.3×10^{6}	2.0×10^{5}	2.7×10^{2}	16	55	6	8	5	6	2
Package paper (UV treated) (P')	41	25	3	0	1	4	1	1	0	0
After packaging (UV treated) (F_4')	4.3×10^{4}	3.4×10^{3}	1.1×10^{2}	0	7	0	1	0	0	0

Altogether 13 steps in milk chain of Dairy Development Corporation, *Balaju* were taken for the study. Microbial counts were found increasing from farm level to just before pasteurization. Higher microflora in farm level revealed the contamination from milch animal, equipment used, milking method applied and from handlers. Increment in bacterial count for raw milk after farm level might concern with the use of unsanitized and uncleaned milk cans and equipments, polluted water supply, unhygienic handlers, microbial growth and inadequate chilling. Pasteurization of milk reduces the microbial count.

Milk examined just after pasteurization was found to be decreasing till homogenization step and then increased. Milk after packaging had showed a higher microbial count than in other steps of pasteurized milk. When processed milk reached at consumer level, the bacterial load was found to be increased to unacceptable level than the standard prescribed by Nepal food standard and WHO. Most of the pasteurized milk samples were found to have bacteria and yeasts/ mold. The increment in total plate count indicated poor hygienic condition prevailing, higher Gram-negative bacteria indicate the higher count of pathogenic bacteria. Higher Coliform count revealed the post pasteurization contamination. Faecal Coliform

indicates faecal contamination (more indicative then Coliform but less than *E. Coli*).

Total Plate Count

In summer season, total plate count for raw milk sample was found to be the highest (1.5×10^{10} cfu/ml) before pasteurization step (F_2 level), and lowest count (2.8×10^7 cfu/ml) from the first sampling step (M sample). Total plate count increased in Evening milk sample (E), Milk producers association (MPA) level, Chilling vat (V), Tanker before Pasteurization (T), sample after packaging (F_6) and at Consumer level (C). Pasteurized milk showed the lowest plate count 1.0×10^6 cfu/ml at F_5 level (before packaging) and highest count (5.7×10^6 cfu/ml) at consumers' level.

Similarly in winter, the highest total plate of raw milk sample was 3.5×10^8 cfu/ml at F_2 point (before pasteurization step) and lowest count i.e. 3.6×10^8 cfu/ml was at M (the first sampling site). Pasteurized milk samples at F_4 step (Homogenization) and F_5 step (sample before packaging) had the lowest count i.e. 1.2×10^5 cfu/ml. The count increased and reached 2.3×10^5 cfu/ml at C (Consumers level). The patterns of microbial count indicate the critical control points (CCPs) in milk chain for TPC. CCPs for raw milk are at E (Evening milk sample), MPA (Milk producers association), Chilling vat (V), (Tanker before transportation) T, F_0 (Tanker after transportation) and F_1 (Raw milk storage tank) and F_6 (sample after packaging) for pasteurized milk.

Gram Negative Bacterial Count

In summer, Gram negative bacteria in raw milk increased from 1.3×10^5 cfu/ml at M (at first sampling point) to 5.4×10^7 cfu/ml at F_2 (before pasteurization step). Gram negative bacteria in pasteurized milk were found to be the lowest i.e. 1×10^2 cfu/ml at F_5 (sample before packaging) and the highest count (3.8×10^2 cfu/ml) at C (Consumers level). The critical steps where Gram negative bacteria increased were E (Evening milk collection), MPA (Milk producers association), T (Tanker before transportation), F_1 (Raw milk storage tank), F_6 (sample after packaging) and C (at consumers level).

While in winter highest Gram negative bacterial count was 2.5×10^6 cfu/ml at F_2 (before pasteurization stage) and lowest count was 4.4×10^4 cfu/ml at M (at first sampling point). Pasteurized milk had the lowest Gram negative bacterial count. i.e. 1.0 cfu/ml at F_4 (Homogenization) and F_5 (sample before packaging) and 16 cfu/ml as the highest count at C (at consumers level). The critical steps in winter season for Gram negative bacterial count were E (Evening milk collection), MPA (Milk producers association), V (Chilling vat), F_0 (Tanker after transportation) F_1 (Raw milk storage tank) and F_6 (sample after packaging).

Similarly, regarding the contaminants in summer can and water of chilling centre were found to be 4.7×10^2 and 1.0×10^4 cfu/ml Gram negative bacteria respectively while contaminants of pasteurized milk BW (Boiling water), TW (tap water), P (Package paper) and A (Air) found to contain no colony, 2 cfu/ml, 5 cfu/ml, 4 cfu/cm^2 and 5 cfu/plate respectively. While in winter 4.0×10^2 cfu/cm and 1.7×10^3 fu/ml of Gram negative bacteria were enumerated from can sample and water sample, respectively. Pasteurized milk showed 2, 5 and 5 cfu/plate for FW (Feed water), TW (tap water), P (Package paper) and A (Air) respectively.

Coliform Count

In summer the highest Coliform count in raw milk reached 5.5×10^6 cfu/ml at F_2 level (before pasteurization) and lowest count (1.6×10^4 cfu/ml) at M sample (first sampling site). Pasteurized milk had the lowest count 40 cfu/ml at F_4 (Homogenization step) and the highest count 1.5×10^2 cfu/ml at C (Consumers level). The critical control points for Coliform bacteria were E (Evening milk sample), MPA (milk producers association level), T (tanker before transportation), F_1 (raw milk storage milk), F_6 (sample after packaging), and C (at consumers level). While in winter raw milk had 5.0×10^5 Coliform as the highest count at F_2 and 8.7×10^3 as the lowest count at M point. Pasteurized milk did not show any colony at steps F_3 (sample after pasteurization), F_4 (Homogenization) and F_5 (sample before packaging) but 13 cfu/ml Coliform count were found at C step. The critical control points for Coliform in winter season were E, MPA, T, F_0, F_1, F_2, F_6 and F_7.

Regarding the contaminants, in summer 1.1×10^2 cfu/cm Coliform was found in Milk Can and 1.7×10^3 cfu/ml Coliform in water at chilling centers. TW, P and A were found to contain 4 cfu/ml, 2 cfu/cm^2 and 10 cfu/plate, respectively. While in winter 1 cm^2 area of can was found loaded with 65 cfu and 1 ml of chilling centre water had the count 1.8×10^2 cfu. TW, P and A, contaminants of pasteurized milk, showed 4 cfu/ml, 2 cfu/cm^2 and 1 cfu/plate, respectively while others, i.e BW and FW had no any colonies.

Faecal Coliform

In summer highest faecal Coliform count in raw milk was 1.8×10^5 cfu/ml at F_2 (before pasteurization step) and the lowest count was 4.1×10^3 cfu/ml at M (first sampling site). The highest count in pasteurized milk was at consumer level i. e 47 cfu/ml and steps F_4 (Homogenization step) and F_5 (sample before packaging) had the lowest count of 7. Considering the increasing number of faecal Coliform, the following were critical control points-E, MPA, T, F_0, F_2, F_6, F_7 and C. While in winter highest faecal Coliform count in raw milk was 4.1×10^4 cfu/ml and lowest count was 2.1×10^2 cfu/ml at steps F_2 and M, respectively and no faecal Coliform were investigated at F_3, F_4 and F_5. Hence, the critical control points were E, MPA, V, F_0, F_1 and F_6.

Similarly regarding the contaminants, in summer 38 cfu/cm^2 and 3.8×10^2 cfu/ml faecal Coliform were enumerated, respectively from can and water used for raw milk. Pasteurized milk was found to contain 1 cfu/cm^2 and 4 cfu/plate, respectively in TW, P and A. While in winter Milk Can and water contained 2 cfu/cm^2 and 56 cfu/ml of faecal Coliform. TW, P and A showed 1 cfu/ml, 2 cfu/cm^2 and 1 cfu/plate, Coliform, respectively.

Yeast and Mold

In summer step, F_2 had the highest yeast and mold count of $1.3x10^5$ cfu/ml and step M the lowest count $5.8x10^3$ cfu/ml for raw milk whereas pasteurized milk was found to be contained 2 cfu/ml as the lowest count at F_2 and 30 cfu/ml as the highest count at C. So the critical steps for summer season for yeast and mold are E, MPA, F_2, F_6, F_7 and C. While in winter, the highest yeast and mold count was $3.8x10^4$ cfu/ml and the lowest count was $1.7x10^3$ in the steps F_2 and M, respectively. Pasteurized milk at step F_4 and F_5 contained no yeast and mold colonies and it had 2 cfu/ml as the highest count at consumer level. The critical steps in winter season are M, MPA, V, T, F_1, F_2 and F_6.

Similarly regarding the contaminants in summer, Milk Can and water found to contain 22 cfu/cm^2 and $3.1x\ 10^2$ cfu/ml yeast and molds at chilling center level. No contamination was shown from BW, FW, TW and P in pasteurized milk while air showed a heavy contamination i.e . 64 cfu/plate. While in winter Milk Can and water were found to contain 2 cfu/cm^2 and 30 cfu/ml of yeast and mold, respectively. BW, FW, TW had no contamination over pasteurized milk, while P and A showed little contamination of 1 cfu/cm^2 and 5 cfu/plate.

Contaminants

In summer, raw milk at MPA level was found to have contaminated from can and at chilling centers from water. Can was found to have contained $8.3x10^3$ cfu/cm^2 and water was found loaded with $1.3x10^5$ cfu/ml. Pasteurized milk was found to be contaminated with feed water, tap water, packaging paper and air. BW (Boiling water), FW (Feed water), TW (Tap water), P (Package paper) and A (air) were found to contain 2, 59, 87 cfu/ ml, 74 cfu/cm^2 and 86 cfu/plate, respectively.

Percentage of Contamination for Pasteurized Milk

Microfloras in raw milk were Coliform (33%), Micrococcus (22%), and Corynebacterium (18%), and in pasteurized milk were Micrococcus (32%), Corynebacterium (20%) and Coliform (17%).

Fig 1 shows the percentage of contamination with different bacterial groups. The highest percentage of contamination was occurred from TW. It has been followed by packaging paper and the third number was occupied by FW. This sequencing i.e. Tap water (TW)> Package paper (P)> Feed water (FW) is shown almost same over TPC, Gram negative count and Coliform count.

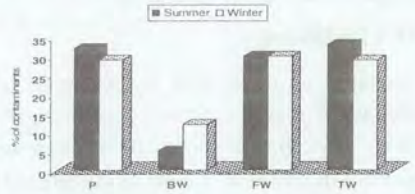

Fig 1: Percentage of contaminants from pasteurized milk in summer and winter season

(P = Package paper, BW = Boiling Water, FW = Feed water, TW = Tap water)

Types of Microorganisms found in Milk

Milk samples were found to be contained various types of microorganisms. The organisms isolated and identified among the organisms which were found in the largest number and 3rd largest number. It was known after their identification that those bacteria were the Coliform, Micrococcus and Corynebacteria. Sometimes, Bacillus and Staphylococcus were found to form the colonies in the largest number. The percentages of main group of bacteria found in raw and pasteurized milk are shown in Table 2.

Table 2: Percentage of bacteria in raw and pasteurized milk

Group of bacteria	*Raw milk (%)*	*Pasteurized milk (%)*
Coliform	33	17
Micrococcus	22	32
Corynebacteria	18	20
Others	27	31

Conclusions

The assessment of microbiological quality system in milk chain of Dairy Development Corporation, *Balaju*, drew attention for a systematic monitoring of microbial, chemical and physical quality of milk in the various stages of milk chain carefully. Hazard analysis critical control point (HACCP) system needs to be implemented effectively in milk chain to improve the existing microbiological quality of milk.

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Study of Actinomycetes Isolates by Randomly Amplified Polymorphic DNA-Polymearse Chain Reaction (Rapid-PCR) from Soil Samples of Lukla of Everest Region

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A random amplified polymorphic DNA fingerprinting assay has been optimized and used to study actinomycetes isolates. The technique can be troublesome and time consuming to establish due the essentially empirical approach to optimization. By standardization of certain parameters and use of a commercially available PCR buffer, a particularly promising primer was identified and a RAPD condition for a highly discriminatory and reproducible characterization of actinomycetes isolates was achieved. Ten actinomycetes isolates, obtained from RLABB isolated from soil samples from Lukla of Everest region by RLABB scientists were typed by RAPD-PCR to estimate genetic diversity. Twenty random arbitrary-primers (obtained from University of British Columbia) rich in GC content were randomly selected for RAPD-PCR of which primer 262 was found best. Higher polymorphic fragments were found in the range between 700-900 bp. Next to it, the range 400-700 and 1200-1600 bp were also found to be highly polymorphic.

Keywords: Actinomycetes, Rapid PCR, Soil, Everest region.

Introduction

The actinomycetes are Gram positive bacteria having high G+C (>55%) content in their DNA. Actinomycetes were originally considered to be an intermediate group between bacteria and fungi but now are recognized as prokaryotic organisms. The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. Actinomycetes population has been identified as one of the major group of soil population (Kuster, 1968), which may vary with the soil type. The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites. They cover around 80% of total antibiotic product, with other genera trailing numerically; *Micromospora* is the runner up with less than one-tenth as many as *Streptomyces*. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60%; *Streptomyces* spp. accounting for 80% of these (Hopwood *et al.*, 2000). Their metabolic diversity explains the widespread biotechnological exploitation as well as their potential use as genetically engineered microorganisms (GEMS) in industry and Agriculture (Mehling *et al.*, 1995). Due to large geographic variation, there is large variation in soil type and their contents in Nepal and hence it is quite likely that the distribution of antibiotic producing actinomycetes is also variable. This study is carried out to optimize and study the antibiotic producing actinomycetes by RAPD-PCR from higher altitude, Lukla of Everest region, Nepal.

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Materials and Methods

The actinomycetes isolates used in this study were obtained from RLABB isolated by RLABB scientists from the soils of Lukla of Everest region. Actinomycetes from the soil had been isolated by pour plate technique on Starch-casein agar and Glycerol-arginine agar after serial dilution in distilled water. Dry colonies of actinomycetes were selected and isolated. The isolates were then preserved in Glycerol based media and stored at -20°C.

Characterization of actinomycetes:

The actinomycetes isolates were characterized by morphological and biochemical methods. Morphological methods consist of macroscopic and microscopic methods. The microscopic characterization was done by cover slip culture method (Pandey, 2004).The mycelium structure, color and arrangement of conidiospore and arthrospore on the mycelium was observed through the oil immersion (1000X). Various biochemical tests performed were as follows: Casein hydrolysis, Starch hydrolysis, Tween 20 hydrolysis, Urea hydrolysis, Esculin hydrolysis, Acid production from sugar, NaCl resistance, Temperature tolerance.

Template DNA isolations

DNA was isolated as described by Kong *et al.* (2001). Actinomycetes were grown in Starch Casein Agar (SCA) for 5 days at 28°C. Mycelia or spores of actinomycetes were collected by centrifugation of 1.5 ml of cultures at 8000 rpm for 10 mins, washed once with SSC buffer and resuspended in 500ul TE containing 5 mg/ml lysozyme. After incubation at 37°C for 2 h, 75 ul of 10% SDS and 125 ul of 5M NaCl were added to the mixture. The samples incubated in dry ice/ethanol (-20°C) for 3 min, in a 65°C water bath for 3 min and on ice for

10 min. Proteinase K (50 ug/ml) was then added and incubated the mixture at 37°C for 30 min. an equal volume of phenol:chloroform (1:1) was added, and upper aqueous phase was recovered after centrifugation (8000 rpm for 10 min). The DNA was pelleted by centrifugation (12000 rpm for 20 min at 4°C) after adding 0.1 volume of 3M sodium acetate (pH-4.6) and double volume of chilled absolute alcohol. After washing once with absolute isopropanol. The DNA was dried and dissolved in 100 ul of TE buffer. Extracted DNAs were quantitated by Spectrofluorometer and diluted upto 20ng DNA/ul in order to feed on the PCR reaction mixture.

PCR Condition

PCR conditions were set up as described by Sahukhal (2006). Twenty GC rich RAPD primers (10-mers) of arbitrary sequence (Table 3) obtained from University of British Columbia, Canada, were initially screened for the ability to produce discriminatory polymorphisms. RAPD-PCR mixture was set up that contained 20-50 ng of genomic DNA, 40 pmol of primer, 1 U of Taq polymerase (Bangalore GENEI.), 200 uM (each) deoxynucleoside triphosphate, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$ and 1% DMSO. Each reaction mixture was overlaid with 25 ul of mineral oil and amplified with a Perkin-Elmer Cetus DNA Thermal Cycler model TC-1 as follows: (i) Initial denaturation, 1 cycle consisting of 5 min at 94°C and (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 2 min at 36°C, and 3 min at 72°C, followed by a final extension step at 72°C for 10 mins.

RAPD products were separated by agarose gel electrophoresis (1 %) with 1X TAE buffer for 2 hrs. Molecular size standards (λ DNA *Hin*dIII digest and ϕ X 174 phage DNA type II digest) were also included in each gel, photographed by the Fototdyne camera using Polaroid film (Porplan 667). The RAPD fingerprints were analyzed visually and the Molecular size of each bands migrated were calculated by plotting Standard curve (Log of molecular weight *vs* distance traveled) of the standard DNA ladders.

Calculation of Discriminatory index value (D)

The Discriminatory index value (D) was calculated using the formula:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} x_j(x_j - 1)$$

Results and Discussion

Colonial and biochemical characteristics

The colonial characteristics and biochemical characteristics of the actinomycetes isolates are given in Table 1 and 2.

Table 1: Colonial characteristics of isolated actinomycetes

Serial No.	*Isolate Code*	*Color of Colony*
1	8.7	Deep brown, rough
2	8.9	Creamy
3	8.15	Creamy, Black centered
4	11.1	Yellowish
5	11.2	Bluish
6	13.1	Deep brown
7	13.2	Bluish
8	15.2	Bluish
9	15.5	Creamy
10	18.3	Creamy

Effects of reaction buffer ($MgCl_2$) on RAPD

Insufficiency of Mg^{2+} is known to reduce the yield of amplification products. Increasing the $MgCl_2$ concentration from 1·5 to 3·5 mmol±1 increased the yield of product. Increasing the pH did not appear to have much effect on the profiles obtained when the $MgCl_2$ and KCl concentrations were optimal. However, at less than optimal concentrations, pH did affect PCR product yield.

Effect of template DNA on RAPD

Amplification was performed using primer 261 with different dilutions of phenol-extracted DNA as template. DNA (20 ng) added to reactions gave optimal amplification. Higher DNA concentrations showed no improvement in amplification and a higher background, while lower concentrations did not support amplification of some high molecular weight amplicons.

Effect of DMSO on RAPD

Without using DMSO smeared bend with very high number of unspecific bands were produced that were difficult to compare. In some cases DNA bands were not observed without using DMSO. DMSO concentrations ranging from 1 to 5% were used. Reaction containing 1% DMSO produce best result indicating that adding DMSO is beneficial for RAPD-PCR of the actinomycetes isolates.

Identification of discriminatory primer(s) for RAPD analysis.

Of 20 randomly chosen primers, two primers (261 and 262) with GC% - 70-80, were found to amplify genomic DNA fragments with reproducible polymorphisms suitable for typing actinomycetes isolates. Primer 261 with GC% 70 was found to produce more polymorphic bands than primer 262 and was used to obtain RAPD profiles of some selected actinomycetes isolates(Table 3). The D value was found to be 0.9555.

RAPD fingerprinting of the Actinomycetes isolates

The RAPD-fingerprint of 10 actinomycetes with primer 262 isolates is given in Fig 1 and their calculated molecular size is given in Table 4. Of the total amplified products, maximum

Table 2: Biochemical Characteristics of the Isolates

S No.	*Isolate Code*	*Hydrolysis tests*			*Nitrate reduction test*	*Citrate test*	*Catalase test*	*Sugar Utilization Tests*					
		Starch	*Tween-20*	*Urea*				*Glu*	*Fru*	*Lac*	*Man*	*Suc*	*Mal*
1	8.7	+	+	+	+	+	+	-	+	+	+	-	+
2	8.9	+	-	-	-	+	+	+	-	-	-	+	-
3	8.15	+	+	+	+	+	+	+	-	-	-	+	+
4	11.1	+	+	+	+	+	-	+	-	-	-	-	+
5	11.2	+	-	+	+	+	+	+	+	-	+	-	+
6	13.1	+	+	+	-	+	+	-	+	-	-	-	-
7	13.2	+	+	+	+	+	+	+	+	-	+	+	-
8	15.2	+	-	+	+	+	-	+	+	+	+	+	+
9	15.5	+	+	+	+	+	-	-	+	+	-	+	-
10	18.3	+	+	+	+	+	-	-	+	+	-	+	-

- = negative, + = Positive

Glu= Glucose, Fru= Fructose, Lac= Lactose, Man= Mannose, Suc= Sucrose, Mal= Maltose

Table 3: RAPD primers producing reproducible polymorphisms with Actinomycetes

S.N	*Primer*	*Sequences of primers (5' to 3')*	*GC% of primers*	*No of bands produced*
1	261	CTGGCGTGAC	70	8
2	262	CGCCCCCAGT	80	6

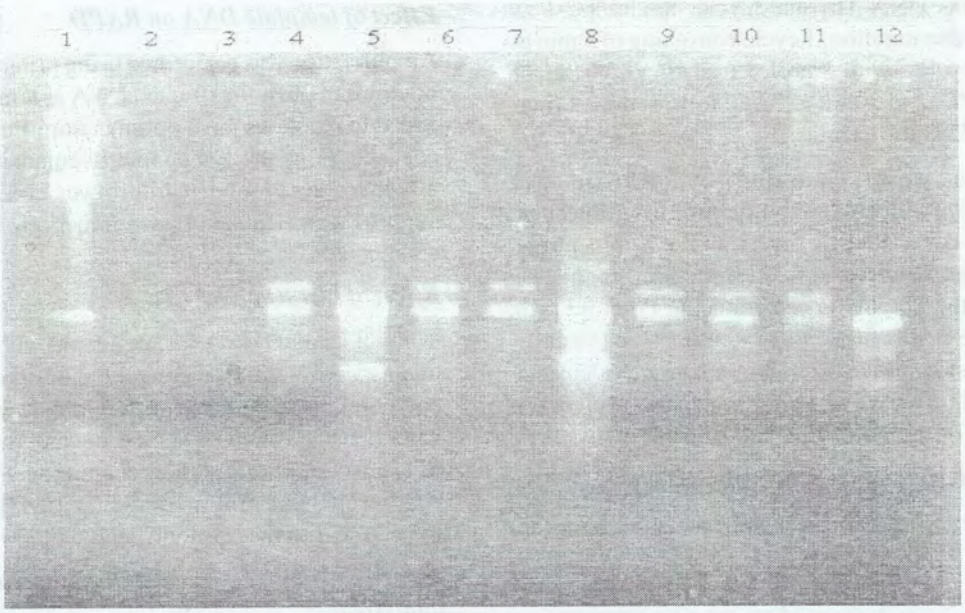

Fig 1: RAPD profiles of actinomycetes isolates using primer 262 Lane 1: λ DNA marker HinIII digest, Lane 2-3: Negative control Lane 4-12: isolates 8.7, 8.9, 8.15, 11.1, 11.2, 13.1, 13.2, 15.2 and 15.5 respectively

Table 4: RAPD types of Actinomycetes from lukla region of Everest

S. N.	*Codes of the isolates*	*No. of RAPD Bands Produced*	*Number of base pairs (bp)*
1	8.7, 8.13	4	935, 499, 414, 279
2	8.9	4	888, 414, 316, 241
3	8.15	63	935, 499, 414
4	11.1, 13.1	2	414, 316
5	11.2	5	888, 668, 414, 263, 163
6	13.2	3	888, 414, 316
7	15.2	4	11382, 888, 519, 414
8	15.5	3	414, 296, 222

number of the product size ranges from 400 bp to 500 bp was found highest followed by product size 200 bp to 300 bp, 300 bp to 400 bp and 800 bp to 900 bp as shown in Table 5.

Discriminatory index value (D)

Of 10 isolates typed, 8 different types of genetically polymorphic isolates were found. The D value was found to be 0.9555

In this study the PCR was used to amplify randomly primed genomic DNA from actinomycetes isolates using a protocol optimized in terms of reaction buffer ($Mgcl_2$), template concentration, template preparation method and choice of primer. The reaction mixture contained 20-50 ng of genomic DNA, 40 pmol of primer, 1 U of Taq polymerase, 200 uM (each) deoxynucleoside triphosphate, 10 mM Tris-Cl (pH 8.3), 50

Table 5: Fragment length polymorphisms among the Actinomycetes isolates

Range of fragment length (bp)	*Total number of bands produced*	*Range of fragment length (bp)*	*Total number of bands produced*
100-200	1	600-700	1
200-300	5	700-800	4
300-400	4	900-1000	3
400-500	11	11000-12000	1
500-600	1		

mM KCl, 3 mM $MgCl_2$ and 1% DMSO and was amplified with a Perkin-Elmer Cetus DNA Thermal Cycler model TC-1 as follows: (i) Initial denaturation, 1 cycle consisting of 5 min at 94°C and (ii) 35 cycles, with 1 cycle consisting of 1 min at 94°C, 2 min at 36°C, and 3 min at 72°C, followed by a final extension step at 72°C for 10 mins. This reaction mixture was found to produce reproducible results. When setting up a PCR facility all usual precautions to prevent contamination must be taken. In this study, pure cultures of actinomycets isolates were processed aseptically to extract DNA template for RAPD-PCR. All the parameters that can influence the efficacy of PCR were considered during optimization of the procedures. Special consideration was focused to optimize final $MgCl_2$ concentration in the reaction mixture as it is of prime importance for the activity of the Taq polymerase. 3.0 mM $MgCl_2$ per 25¼l reaction mixture was found to be optimal for the actinomycetes DNA preparations. In this study, 1% final concentration of DMSO was also used. Various authors recommended DMSO to improve amplification efficiency (higher amounts of products) and sp/ecificity (no unspecific products) of PCR. DMSO concentrations varying between 5-10% v/v were also tested but no such remarkable effects were found.

The ratio of DNA template to primer is one of the most critical factors to consider when optimizing the PCR; therefore, a range of DNA concentrations should be titred against a fixed primer concentration for each DNA extraction protocol to obtain the ideal conditions (Tyler *et al.*, 1997). Although it has been suggested that phenol-extracted DNA can aid reproducibility, using whole cell template may save time during investigation of large samples. It seemed that whole cell template is a good substitute for phenol-extracted DNA. However, comparison of phenol-extracted DNA template profiles and boiled whole cell template profiles by Madico *et al.* (1995) revealed that profiles were usually similar but some differences occurred as a result of damage to large DNA fragments during boiling. This may also lead to a lack of reproducibility as damage to the DNA is a random event. Hilton *et al.* (1997) found that ethanol-treated whole cell template gave profiles almost identical to those obtained from phenol-extracted DNA. However, reaction products were less stable and profiles smeared if not analysed within 24 h post amplification.

Of 20 primers tested, 2 primers (261 and 262) with GC% 70-80, were found to amplify the target sequences with reproducible polymorphism to differentiate the actinomycetes strains. PCR products were visualized by ethidium bromide staining (0.2-0.5 ug/ml of gel) of electrophoretically separated DNA in agarose gel. Fingerprints were recorded as banding patterns and comparisions were made by visual inspections. In this study DNA bands and R_f values were interpreted using standard scales (» DNA HinIII digest). Each primer amplified the DNA bands ranged from 200 bp to 12 kbp. The bands were found to be reproducible for different independent DNA preparations from respective actinomycetes strains.

Primers 262 yielded RAPD patterns that were unique to strains of the actinomycets isolates to be differentiated subsequently. Of the total amplified products, maximum number of the product size ranges from 400 bp to 500 bp was found highest followed by product size 200 bp to 300 bp, 300 bp to 400 bp and 800 bp to 900 bp as shown in Table 5. Isolates 8.7, 8.13 and 11.1, 13.1 showed similar band patterns. Similar band patterns suggest that the strains are closely related to each other within each group; however, the data must be interpretative with caution since PCR bands of similar size do not necessarily mean that the molecules are identical in sequence. DNA product size of 414 bp was found in all actinomycete isolates suggesting certain similar characteristics among them that may antibiotic producing but must be confirmed by some other techniques. Product size of 888 bp was also found common in many isolates. As the strains were isolated from high altitude mountain region of the country, the maximum amplified genomes may represent antibiotic producing gene or cold tolerant genes common to all but must be confirmed by sequencing. However, for the

best knowledge, such study for cold tolerance were not found yet and the data were not compared to any reference but predicted to contain common or consensus bands (or may be sequences) for cold tolerance so as to adopt the organisms in the given ecological niche.

Vogel *et al.* (1999) tested the usefulness of genomic typing methods *viz.* RAPD analysis and ribotyping with conventional serotyping for three collections of well defined clinical *E. coli* isolates and found that RAPD has the highest discriminatory capacity. Similarly, Sarkar *et al.* (2002), exploited the RAPD-PCR fingerprinting analysis and showed a high level of diversity of *Bacillus* spp. and related genera. According to Vogel *et al.* (1999) for both RAPD and ribotyping, banding patterns with a difference of e"2 band were considered to represent different strains while isolates with <2 bands difference were regarded as the same strain. In order to determine the index of discrimination (Hunter, 1990; Hunter & Gaston, 1998), all the 10(*N*) actinomycetes strains were classified into 8(*s*) types with two sets having similar band patterns (8.7, 8.13 and 11.1, 13.1). Thus in this study, the RAPD-PCR, used to categorize the actinomycetes strains isolated from Lukla region of Nepal, was found to discriminate the organisms with 95.5% confidence (D = 0.9555) and the polymorphisms set by RAPD to quantify the genetic diversity of actinomycetes can be confidently defined as rich one.

Conclusion

Of the 20 randomly selected primers, primer 262 was found best to study actinomycetes by RAPD PCR. This primer yielded RAPD patterns that were unique to strains of the actinomycetes isolates to be differentiated subsequently. Higher polymorphic fragment was found in the range between 700-900 bp, 400-700 bp and 1200-1600 bp. RAPD-PCR successfully discriminated the organisms with 95.5% confidence (D = 0.9555) and thus can be concluded that the polymorphisms set by RAPD to quantify the genetic diversity of actinomycetes can be confidently defined as rich one.

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News and Events

SOM NATH KHANAL*

Computerized Milk Collection System in Cooperatives.

Production and collection of fresh milk is being more and more commercialised in Nepal through continuous and extensive efforts of milk producers cooperative associations. The cooperatives are not only trying to provide better services to satisfy the needs of their dairy farmers, they are also trying positively to improve the quality of the fresh milk they produce. Installation of Computerised Automatic Milk Collection System has shown its positive impact for improving quality of milk. The system comprises of a digital milk-weighing pan; milk analyser for fat, SNF, adulterants and other components; and a software aided computer system. Devdaha Milk Producer Cooperative Association (DMPCA) situated in Devdaha Village Development Committee of *Rupandehi* district is the first of such cooperatives to use such a system in the country. The system has become an appropriate alternative to the time consuming and rather less transperant conventional methods. It is gaining its popularity amongst the farmers, other cooperatives and dairies because of its affordable price, transperency, quick, convinient and accurate results, computerised billing and easy operation. The system is developed in India and costs around four lakhs Nepali rupees, which can be considered as affordable for the well-established dairy cooperatives and milk processing industries of the country.

Hetauda Dairy Produces Sweetened Condensed Milk

Condensed milk has been considered as a major dairy product since decades; however, its production in Nepal was started only after the establishment of Hetauda Dairy industries P. Ltd. in 2002 at *Newarpani* near *Hetauda,* a city of *Makawanpur* district. The industry has exclusively engaged in production of Sweetened Condensed Milk of quality to satisfy the Nepalese Standard. The factory has a processing plant made in China having the capacity of 4 tonnes of condensed milk per day. The product is available in the market under the brand name of NESCOMILK in different size of packagings viz. 397 g and 7.5 kg laquered tin cans. The product is also available in larger size packages as per the demand of the consumer. The major consumers of the product are the general public of different urban region of the country and the biscuits and confectionary industries, which, otherwise, had to depend on the imported product of such kind. The industry claims the shelf life of the canned product to be 6 months from the date of manufacture. For being the relatively longer storable dairy product, this condensed milk production can be expected to reduce the milk holidays of winter season in *Chitawan* and *Makwanpur* districts from where it procures the required raw milk.

DDC Acquired HACCP.

The Kathmandu Milk Supply Scheme (KMSS), *Balaju*, *Kathmandu* and Milk Products Production and Supply Scheme (MPPSS), *Lainchaur* of Dairy Development Corporation (DDC) in Nepal have recently acquired the HACCP waranty certificate of food safety for their particular scope of productions. In KMSS, HACCP warranty certificate for manufacturing and supply of Pasteurized milk, Table butter and Ghee, accredited by Joint Accredition System of Australia and Newzealand (ACC No. H 2690803 NA) has been issued by the corporate office of International Certification Ltd. Auckland, Newzealand on July 2006. In MPPSS *Lainchaur*, the HACCP warranty certificate for manufacturing and supply of Ice cream and Dahi was issued by TUV. NORD of Germany on March 2007. From this certifications, DDC has become the second industry of food manufacture in Nepal to acquire HACCP. Earlier to DDC, Instant Meal P. Ltd. of *Sunsari* district acquired HACCP certification for baby food from Det. Norske Varitas (DNV) of *New Delhi*, India in 2003.

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