Parasitological and selected microbiological safety aspects of Nham (Thai-style fermented pork sausage) were evaluated. Pork meat from pigs experimentally infected with *Trichinella spiralis* was divided into 4 portions; 3 portions were frozen at -23°C for 12, 18 and 24 hours before they were made into Nham. The formula for fermented sausage included 3% w/w NaCl and garlic, 0.015% each of NaNO₂ and NaNO₃, and either 1.0 or 1.5% w/w commercial starter culture. Fermentation was at 30°C for 96 hours. The higher level of starter culture resulted in faster acid production but no difference in the maximum number of lactic acid bacteria attained. Infected pork, after being frozen for 24 hours, was still found to cause a low level of parasitism. However, the corresponding fermented samples were noninfective. Use of 1.0% starter culture and 12 hours freezing of meat prior to grinding rendered trichinae larvae noninfective after 2 days of sausage fermentation.

*Staphylococcus aureus* (10^3 cells per g) and *Escherichia coli* (10^6 cells per g) were introduced into the ground pork in a series
of experiments. Starter culture levels used were 0.75% and 1.5% by weight. Fermentation without starter culture stabilized the numbers of *E. coli* but permitted slow multiplication of *S. aureus*. Adding starter culture at either level increased the rate of acid production during the initial days, resulting in a faster drop in pH and a decline in numbers of both *S. aureus* and *E. coli*. After 36 and 48 hours, viable *S. aureus* were not recovered from products with 1.5% and 0.75% starter culture, respectively. *E. coli* disappeared from sausages with 1.5% starter culture after 96 hours but persisted at low numbers when the lower level of starter culture was used.

The rapid direct plating technique (RPT) for enumeration of *E. coli* was compared to the two standard methods generally used: plating on Violet Red Bile Agar (VRBA) and Most Probable Number (MPN). Total coliforms from 43 fermented pork sausages were enumerated. The RPT gave data highly correlated to those from VRBA but with a significantly lower correlation to MPN. The regression line of log10 coliform counts from the RPT and VRBA had a slope of 0.76 and an intercept of 0.98. The MPN assay gave significantly higher (P=0.05) numbers than either the VRBA or the RPT.
Thai-Style Fermented Pork Sausage (Nham): Parasitological and Microbiological Safety

by

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THAI-STYLE FERMENTED PORK SAUSAGE (NHAM):
PARASITOLOGICAL AND MICROBIOLOGICAL SAFETY

CHAPTER I

INTRODUCTION

In a developing country like Thailand, fermentation is one of the most economical methods of producing and preserving food. Thai fermented pork sausage (Nham) is a high protein food, which is well known to Thai people, particularly in the Northeastern region. It consists of fresh pork meat that is trimmed of fat, ground, mixed thoroughly with salt and garlic and traditionally wrapped in small banana leaf packets. In commercial production, plastic bags or tubes are widely used and a starter culture added to insure the success and uniformity of the product. Regulated levels of nitrate and nitrite salts are also added for color improvement. The flavor and texture of Nham is the result of lactic acid fermentation, which is comparable to Western sausages fermented with pediococci. Since the pork is generally not cooked before consumption, there is a possibility of the product causing trichinosis in the consumer. An outbreak of trichinosis that occurred in Chiang Mai Province, Thailand, in 1975 was attributed to Nham (Khamboonruang et al., 1978). In a survey of the prevalence of trichinosis in Thailand, it was concluded that locally raised pigs were the major reservoir host for human infections (Dissamarn et al., 1980).
The hygiene of Nham production is also in question from the standpoint of food safety because it mostly occurred at the household level, where the level of sanitary practice varies greatly. Prevalence of heat-stable II enterotoxigenic *Escherichia coli* (ST-II ETEC) in Thailand was investigated recently and suckling pigs with diarrhea were the major reservoir found (Echeverria et al., 1984). This study also found ST-II ETEC infrequently in humans with or without gastrointestinal symptoms and in collected drinking and bathing water.

Worldwide, *Staphylococcus aureus* has been responsible for food poisoning incidents which traced back to fermented sausage such as Genoa salami (USDHEW, 1971). *S. aureus* can multiply and produce toxin during the initial stage of fermentation, given the proper conditions. Other than *E. coli* and *S. aureus*, which are commonly associated with raw ground meat, there is also the possible presence of salmonellae and clostridia. The prevalence of salmonellae in meats varies considerably. Of 560 fresh pork sausage samples studied (Surkiewicz et al., 1972), 28% were contaminated with low numbers of *Salmonella*, whereas only 0.4% of 735 fresh beef patties contained *Salmonella* (Surkiewicz et al., 1975). The prevalence of *Clostridium botulinum* spores in fresh and semipreserved meat is low (< 1%), and, when spores are detected, they are always present in very low numbers; less than 4 per kg (Riemann et al., 1972; Roberts and Smart, 1976). Because of its ubiquitous distribution in nature (Genigeorgis, 1975), *Clostridium perfrin-
gens is expected to be in most meat and dry ingredients, but in small numbers.

This study evaluated the potential for rendering trichinae larvae noninfective and killing selected pathogens during Nham fermentation in a typical small scale production. Secondly, other factors such as levels of starter culture, pH and freezing times, singly or combined, to render the product safe for consumption were explored.
CHAPTER II

REVIEW OF LITERATURE

The flavor and texture characteristics of Nham depend on a natural lactic acid fermentation, which results in sausage-like flavor similar to Western sausages fermented with pediococci. This sausage usually has a short processing and aging time (1-4 days). The $A_w$ is high since it does not go through a drying process and water content is more than 60%. On the fourth day the sausage should be ready to eat and will feel firm when pressed between the fingers and have a pH of 4.6 ± 0.1 (Yanasugondha, 1977). It should remain in good condition for another 24 to 48 hours without refrigeration. However, refrigeration can prolong this quality for three weeks. If Nham is left at room temperature for seven days, it becomes spoiled and no longer fit for consumption. It was described by Yanasugondha (1977) as soft and crumbly when pressed between the fingers, slimy feeling on the surface, and changed in organoleptic qualities due to off-flavor and odor. The pH of spoiled sausage (4.8-5.0) is slightly higher than the finished product. Change of texture resulted from reaction of microbial enzymes causing proteolysis and/or lipolysis of the protein matrix. Products such as amines and short chain fatty acids were formed (Reuter, 1975; Stamer, 1975). Off-flavors could be caused by minor fermentation products of the acid-tolerant group of lactobacilli and leuconostocs which were still active and
utilized organic acids previously formed. Slime-producers in ham-curing brine were identified as *L. plantarum* and *L. casei* and also leuconostocs (Deibel and Niven, 1959). These microorganisms were also found in the late stage of Nham fermentation (Yanasugongdha, 1977). Refrigeration will slow down the activity of these microorganisms, thus prolonging the shelflife of Nham. Since Nham has a high \( A_w \), biologically produced acid is relied upon for enhanced keeping quality. In today's homemade sausage, a dry starter culture is used to speed up the fermentation process and control the development of the microflora, therefore increasing uniformity of the finished product, minimizing failure and improving flavor. The starter culture comes under the name Nham Powder and is available at local grocery stores.

**Fermentation Process**

Success in making this fermented pork sausage is dependent on the type of microorganisms developing in the raw, comminuted meat. The microbial load of raw meat is usually about \( 10^3-10^4 \) per g (Sutherland and Varnam, 1982). Grinding meat increases the meat surface and may increase the microbial load up to \( 10^5-10^6 \) per g. Many different types of microorganisms on raw pork meat are mainly gram-negative bacteria (Gardner et al., 1967; Hall and Angelotti, 1965; Vanderzant and Nickelson, 1969). They are primarily aerobic and facultative microorganisms from hide and skin of the animals, equipment surfaces, personnel handling or just air contact (Ayres,
1955; Newton et al., 1977, Stiles and Ng, 1981). The lactic acid bacteria represent only a small fraction of the flora in uncured meat (Kitchell and Shaw, 1975).

The changes that occur during fermentation of Nham are the result of the activity of enzymes produced primarily by microorganisms that are involved in the fermentation process. The major microbiological changes occurring during natural fermentation of this meat mixture are the shift of the fresh, refrigerated or frozen meat flora from Gram-negative, catalase-positive, highly aerobic flora to a Gram-positive, catalase-negative, and mostly microaerophilic flora. These changes are attributed partly to the selective inhibitory effect of the salts added and partly to the change in the redox potential of the system. The decrease in $O_2$ tension results from tightly wrapping the product and the initial aerobic metabolism of natural flora with subsequent decrease in the redox potential within the sausage. At the same time that aerobic organisms cannot grow, microaerophilic bacteria proliferate under these favorable conditions of reduced oxygen level. Other parameters are temperature and gas composition of the environment, e.g. $CO_2$. The bacterial types usually developing under such condition are lactic acid bacteria which utilize carbohydrates, producing acids and lowering the pH of the system, which causes suppression of undesirable bacteria, including pathogens.

Lactic acid bacteria, mainly *Lactobacillus* species, have been
shown to proliferate rapidly during fermentation of meat (Pederson, 1979). The heterofermentative species of lactobacilli can produce a moderate amount of acid while pediococci produce a greater amount, and the homofermentative species of lactobacilli produce the greatest amount of acid. Homofermentative species of pediococci and lactobacilli convert sugars primarily to lactic acid. Heterofermentative species of leuconostocs and lactobacilli convert glucose to about 50% lactic acid, 25% acetic acid and ethyl alcohol, and 25% carbon dioxide. Homofermentative types tend to give better products of more uniform quality than the heterofermentative types which can cause slimy and discolored sausage (Niven et al., 1949; Chyr et al., 1981).

The specific conditions which promote the subsequent microbiological changes that lead to the right proportion of fermentable products are important in relation to flavor development of specific products. Natural fermentation is generally a mixed culture process. The group that initiates a fermentation will develop until its by-products of growth inhibit further multiplication. During this initial period, other organisms develop and in turn are followed by other more tolerant species. There are definite reasons for this sequence. The smaller the cell, the greater the surface area in proportion to the cell mass and the more rapidly it can take up nutrients from its surroundings (Pederson, 1979). The smaller-celled bacteria, such as Pediococcus and Leuconostoc species, would grow and
ferment more rapidly than the closely related but larger cells of genus *Lactobacillus*.

The use of starter cultures is based upon selecting those species of lactic acid bacteria which are most desirable in the fermentation process. To speed up the fermentation process, to increase uniformity of finished product from batch to batch, to minimize losses and to improve flavor, the use of starter culture in making fermented meats becomes more common, especially in commercial scale production. *Pediococcus cerevisiae* was considered the most suitable for fermented sausage products in the USA (Niven et al., 1958). Its popularity has also increased among European producers in recent years (Erichsen, 1983). Starter cultures that are now available commercially are all combinations of the following main types of bacteria: lactobacilli, pediococci and micrococci. Originally they were available in dry powder form but frozen concentrates have become increasingly popular in industrial scale production since it was observed that they are more active and have a shorter lag phase (Erichsen, 1983).

**Health Hazards**

**Parasitological Aspects:** *Trichinella spiralis* and trichinosis

*Trichinella spiralis*, a nematode, represents a hazard to both animal and human health. The pathological symptoms of trichinosis were present quite expansively in a wide geographic distribution long before the parasite was identified as the causative agent by
a German pathologist, Friederich Albert von Zenker in 1859 (Campbell, 1983). Since then, there has been extensive study of every aspect of the parasite itself and the disease trichinosis. Much of the data concerning human foods has been derived from the infected domestic pig because it is the best known and most important source of infection for the human. Also, the human infection was reported historically in records from urban centers or from countries with large ethnic groups whose eating habits included consumption of either raw or under-cooked pork.

Stoll (1947) reported that the human infection rate with *T. spiralis* in the United States was three times higher than that of the rest of the world. The U.S. pork industry has long suffered from the stigma of trichinosis as a disease associated with the consumption of pork. Many countries other than the U.S., that are large consumers and exporters of pork, have required by law that each pig be inspected for *T. spiralis* infection at the time of slaughter. The result has been that the disease has been controlled so well in swine that human infection almost never occurs. In the U.S., where pork consumption is very significant (40.7% of all meat consumed, USDA 1981), an annual mean of 133 new human cases was reported for the 1970-79 decade (Centers for Disease Control, 1980). The U.S. population has been educated over the years about the dangers of trichinosis, but cases still occur, usually from eating country or farm style sausage prepared by ethnic groups (Centers for Disease Control, 1980). Such
sausage has been implicated in approximately 55% of the cases associated with pork, the remainder being caused by chops, bacon, ham and roasts (Juranek and Schultz, 1972). The U.S. Department of Agriculture, through its meat inspection agency (Food Safety and Inspection Service), has established processing procedures which render pork products free of infective trichinae (USDA, 1973). These regulations include: (a) heating pork to at least \(58.3^\circ C\) (137\(^\circ F\)), (b) freezing at specified time-temperature combinations, or (c) curing and drying for specific time periods to destroy trichinae. On January 15, 1986, USDA also approved the use of Gamma radiation as another means to control T. spiralis in fresh pork (21 CFR Part 179, 1986).

According to Zimmermann (1974), an estimated 111,000 swine are infected each year in the U.S., resulting in at least 40 million potential meal exposures. Therefore, although there has been a decline in the infection rate of swine from 0.5% in 5955 swine examined in 1964-1966 (Jefferies et al., 1967) and 0.71% in 1966-1967 (Bair and Etges, 1969) to 0.194% in sows slaughtered at a Federally inspected abattoir in Kentucky in 1976 (Pullen et al., 1977), swine remain a major source of infection for humans. The decline in prevalence rates is influenced by the preponderance of grain-fed midwestern hogs. Juranek and Schultz (1978) found an infection rate of 0.1% in grain-fed pigs (which constituted 98.5% of the U.S. pork supply) and 0.5% in garbage-fed pigs. Although few in number, regional prevalence studies indicate that swine
reared in the eastern U.S. have higher infection rates than do those from the midwest (Schad et al., 1985). This difference was assumed to reflect the greater reliance on the feeding of garbage among eastern hog producers (Zimmermann, 1975).

There is epidemiological evidence that there are other important sources of infection for swine in addition to uncooked garbage (Zimmermann et al., 1962; Rothrock, 1965; Murrell et al., 1984). While pork from infected swine is the most common source of infection, the meat of wild carnivorous animals can also cause infection in both humans and swine. More than 75 species of wild animals from various parts of the world have been reported to be natural hosts of this parasite (Shultz, 1970). It must be accepted that even though it may be possible to control trichinosis in hogs to the point where pork no longer constitutes a threat to human health, wild animals will always remain a possible source of both swine and human outbreaks. Because there are no preslaughter controls for T. spiralis larvae, emphasis must be placed on public awareness and destruction of infectivity of the larvae.

The only information available on trichinosis in Thailand is that on outbreaks of human trichinosis. During 1962-1964, three outbreaks were reported in northern Thailand (Dissamarn and Chai-Ananda, 1966). In the third outbreak in Mae Ngon district involving 258 cases and 15 deaths, the source of infection was a wild pig that had been hunted and shared. Examination of 50 pigs
in the area as well as a few specimens of wild animals proved to be negative. In 1965, 11.43% of hill tribe pigs from three provinces (Chiang-mai, Prae and Nan) were found to be infected. The level of infection varied from 7-5570 larvae per 100 gram muscle. In Prae province, it was reported that 1 out of 12 rats was also positive. However, Dissamarn and Chai-Ananda (1966) concluded there were no reports of human infection because people in this region ate well cooked meat. An outbreak that occurred in Chiang Mai province in 1975 was attributed to raw meat in the form of "lahbor Nham", a favorite dish of northern Thailand (Khamboonruang et al., 1978). To establish the epidemiology of the disease in this province, especially the role of rodents in the transmission step, 1070 rodents were examined, but all proved to be negative. Of the 7598 pig diaphragms examined by digestion, only one diaphragm was positive and at a low level (42 larvae per gram). The epidemiology of trichinosis in Thailand is still unclear due to the rarity of the infection in urban rodents and the lack of extensive surveys of wildlife. However, the hill tribe pig has been shown to be the major reservoir host for human infection (Dissamarn et al., 1980).

**Destruction of T. spiralis Larvae**

In the U.S., destruction of the larvae under the government's program is accomplished by freezing, heating or curing the meat (USDA, 1973), and in the future by radiation. Though freezing can
be utilized to destroy the larvae as in the USDA certification program, viable larvae of *T. spiralis* were obtained from muscle samples of marten, wolverine, polar bear and arctic fox which had been frozen for 5, 6, 12 and 14 months, respectively (Chadee and Dick, 1982). It was suggested that northern isolates of *T. spiralis* may be resistant to low temperature. Mass of host tissue, lipid content of muscle, rate of freezing, characteristics of the freezing process, age of cyst and degree of calcification are some of the important factors in determining survival after freezing (Dick and Belosevic, 1978). The specific recommendations given for freezing are: if meat is less than 6 inches thick, to 15°C for 20 days, -23°C for 10 days or -30°C for 6 days. These freezing recommendations, however, are not expected to apply to wild game meat infected with the Arctic strain. This strain, or *T. pseudospiralis* as some scientists prefer to call it, is poorly infective to domestic swine and has not been identified in a natural hog infection (Murrell et al., 1985).

Extensive studies were carried out by Ransom et al. (1920) to determine the effect of curing procedures on the viability of trichinae in various pork products. Trichinae in hams were rendered inactive by either of these two basic procedures:

(1) cure hams by means of dry salt (4% w/w) for 40-day period at a temperature not lower than 4.4°C, followed by smoking or drying for 10 days at a temperature no lower than 35°C; or

(2) cure hams on the basis of three days' cure per lb of
individual hams (4% salt w/w), followed by 48 hours of smoking at a temperature not lower than 26.7°C and 20-day drying at a temperature not lower than 7.2°C. They concluded that salt and drying temperature were the primary factors affecting the trichinae, the former undermines the vitality probably by dehydration, exerting upon the parasites a direct toxic action. Salt also lowers the resistance of the larvae to heat and thus renders them susceptible to temperatures which normally would not prove fatal.

Gammon et al. (1968) investigated another salt-temperature effect for hams and shoulders for larger scale use. The procedure was that pork was dry-cured at 2 days per lb at 2.2°C; followed by 30 days' hanging at the same temperature for salt equalization; rinsing, drying, smoking for 24 hours at 32.2 - 37.8°C and aging at 23.9°C for four weeks in order to obtain complete absence of infective larvae. Crouse and Kemp (1969) used similar procedures but changed salt equalizing temperature from 2.2°C to 7.2°C for heavy hams and shoulders and found three weeks' aging to be effective in eliminating viable trichinae. Allen and Goldberg (1962) examined the effect of salt alone and reported that trichinae in 1.9 cm cubes of pork containing 3.5, 2.5 or 2% salt retained their infectivity an average of 57, 85 and 107 days, respectively. With ground pork, the average period was decreased to 38, 64 and 84 days. They attributed the difference to the ease of penetration of salt in the ground meat. Olson et al. (1972) reported that salt concentration alone was not effective in
eliminating the infectivity of *T. spiralis* larvae in cured hams and shoulders, rather they stated the combined action of drying time and temperature was the more decisive factor. Other ingredients, such as nitrite, sugar or garlic oil, had no measurable influence on infectivity of the larvae in ground meat, but did cause an earlier loss of infectivity when incorporated into sausage in casings. Water activity (*A_w*) values ranging from 0.949 to 0.904 for the hams were necessary to render *T. spiralis* larvae noninfective (Lotzsch and Leistner, 1979).

**Microbiological Aspects**

A prerequisite for pathogenic bacteria to cause problems in connection with fermented foods is either (1) that they are present in large numbers in the raw material from the beginning or (2) that they are given the opportunity to multiply during the initial stages of the fermentation process. The organisms chosen to be included as potential risks in connection with this fermented sausage study were *Staphylococcus aureus* and *Escherichia coli*.

**Staphylococcus aureus**

In recent years *S. aureus* has attracted considerable attention with regard to the safety of fermented meats. Staphylococci were predominant among the isolates obtained from muscle tissue of beef, pork and lamb carcasses and a large percen-
tage of these staphylococci (77%) were coagulase-positive (Vanderzant and Nickelson, 1969). These staphylococci can grow better than other pathogens under what would be considered adverse conditions such as those prevailing in fermented sausage. *S. aureus*, naturally present or added by contamination during the manufacture of fermented sausage, may grow rapidly during the first 2-3 days of fermentation (Daly et al., 1973). Different types of lactic acid bacteria have been shown to exert strong limiting effects on both growth and toxin production by *S. aureus* (Graves and Frazier, 1963; Haines and Harmon, 1973; Genigeorgis, 1976). *Pediococcus* species were found to inhibit *S. aureus* more effectively than lactobacilli due to the more rapid fall of pH caused by acid production of these bacteria during the early stage of fermentation (Daly et al., 1973; Genigeorgis, 1976). Practical experiments with dry sausage have shown that if lactic acid bacteria are added together with *S. aureus*, the lactic acid bacteria will suppress and completely inhibit the activity of *S. aureus* (Daly et al., 1973; Niskanen and Nurmi, 1976). Excessive growth of *S. aureus* in fermented sausage usually reflects mishandling of raw meat, contaminated equipment and/or poor personnel hygiene (Vanderzant and Nickelson, 1969).

**Escherichia coli**

*E. coli* is one of the natural flora of the gastrointestinal tract of human and warm-blooded animals, therefore its presence is
also common on raw meats. It is used to indicate recent direct or indirect fecal contamination of water supplies, processed or unprocessed foods (APHA, 1965, 1967). In ground beef, *E. coli* type I (IMViC ++--) is one of the predominant *Enterobacteriaceae* (Ng and Stiles, 1978). With good manufacturing practices, *E. coli* type I contamination is generally considered to come from the skin or hide of animals during processing (Ayres, 1955; Hess, 1973; Shooter et al., 1974) and possibly represents both fecal and nonfecal contamination (Newton et al., 1977). In addition, *E. coli* type I in the stationary growth phase survives well in frozen and refrigerated meat (Mackey et al., 1980; Stiles and Ng, 1980) and grows in meats at improper storage temperatures. There has been increasing concern about the presence of *E. coli* in food products because of the ability of some strains to cause foodborne illness (Costin et al., 1964; Bobbs and Mair, 1949). In the review by Gross (1983), the author grouped *E. coli* strains that cause diarrhea as follows:

1. **Enterotoxigenic *E. coli*** (ETEC). They are the group which produce heat-stable or heat-labile enterotoxins which are responsible for some outbreaks of diarrhea among infants in hospital nurseries and are common causes of diarrhea among travellers. The symptoms are described as cholera-like syndrome; watery stool and dehydration, which can lead to high mortality rates, especially in regions with poor hygiene such as the tropics or less developed countries.
(2) Enteroinvasive *E. coli* (EIEC). This group causes symptoms resembling shigella dysentery; i.e. epithelial invasion and intracellular multiplication in the large bowel leading to inflammation and ulceration of the mucosa, fever and diarrhea with blood and mucus in the stools.

(3) Enteropathogenic *E. coli* (EPEC). These strains do not produce any enterotoxin nor do they possess genes coding for enterotoxin production. They also lack the enteroinvasive capacity. However, they are the common causes of infantile enteritis in hospitals and nurseries in western countries and enteritis in tropical countries. They are capable of colonizing the epithelial mucosa of the small intestine and some strains can produce cytotoxin which is distinctive from those of ETEC.

Acute and chronic diarrheal symptoms in humans of all ages frequently are the result of enteropathogenic and enterotoxigenic strains which colonize and subsequently produce toxins in the gastrointestinal tract. Adherence of *E. coli* to epithelial surfaces of the small intestine is mediated by specific, heat-labile surface antigens. These antigens exhibit a fine filamentous or pilus like structure and cross-species reactivity among swine, bovine, sheep and human (Jones and Rutter, 1972; Ørskov et al., 1975; Smith and Linggood, 1971, 1972). Each of the host-specific adherence factors of *E. coli* is plasmid-mediated (Evans et al., 1975; Ørskov and Ørskov, 1966; Ørskov et al.,
The production of enterotoxins by some strains of *E. coli* is also mediated by plasmids (Gyles et al., 1974; Skerman et al., 1972; Smith & Linggood, 1971) that are transferred between strains of *E. coli* in a manner similar to the spread of R-factors of antibiotic resistance (Lacy, 1975). This plasmid-mediated nature of *E. coli* explains the early problems in isolating and identifying their presence in foods (Mehlman et al., 1974) and the difficulty of determining potential pathogenicity of isolates (Sack, 1975; Sojka, 1973). Early workers identified only a few strains (those genetically determined) as pathogenic.

There have been several investigations into the incidence of *E. coli* and enteropathogenic *E. coli* in fermented dairy products (Jones et al., 1967; Lyons and Mallman, 1954; Singh and Ranganathan, 1974). Results from these studies showed that presence of *E. coli* in dairy products usually results from post-pasteurization contamination but whether or not such contaminants are pathogenic is a question to be adequately answered by isolating and identifying enteropathogenic *E. coli* and determining the pathogenicity of the isolates. The lactic acid fermentation in milk controls growth of *E. coli* mainly through lowering of pH and the possible production of antibiotics and other growth inhibitors (Park et al., 1973). In fermented dairy products, survival of *E. coli* is highly variable, depending on starter culture, pH, temperature of storage and composition of the product (Goel et al., 1971; Park et al., 1973; Potashnik et al., 1972;
Wilson and Weiser, 1949). The use of lactic acid fermentation does not necessarily guarantee the safety of the products, as evidenced by growth of *E. coli* in soft-ripened cheese (Fantasia et al., 1975) and an outbreak of foodborne disease traced to imported French cheese (Marier et al., 1973).

**Effects of Fermentation Components on Survival of *S. aureus* and *E. coli***

Salt is used as an ingredient in fermented foods to favor the growth of the desirable microorganisms. At concentrations used in raw sausages (2-4%), common salt (NaCl) is not bactericidal but for some species is bacteriostatic. The tolerance of microorganisms to NaCl differs. Growth of some is inhibited by 2% salt; others are able to grow in saturated salt solution. The growth and metabolism of microorganisms demands the presence of water in an available form. The availability of water in food systems is expressed as $A_w$, which is defined as the ratio of the water vapor pressure of the food to that of pure water at the same temperature. Adding salt reduces $A_w$ in a food by increasing the concentration of solutes in the aqueous phase of the food. When a solution becomes more concentrated, more water molecules are oriented about the added solute molecules, vapor pressure decreases and the $A_w$ falls from its previous value. Survival of vegetative bacteria in the presence of solutes shows no consistent pattern, being markedly influenced by the nature of substances.
The growth of most bacteria, including pathogens, is confined to the $A_w$ range above 0.90. Below this $A_w$, the growth rate and the stationary population or ultimate cell mass decrease, and the length of the lag phase increases. At a sufficiently low $A_w$, which is not the same for all microorganisms, the lag phase becomes infinite and growth does not occur (Scott, 1957). Fermented sausages (not dried) can have an $A_w$ in the range of 0.93-0.98 (Christian, 1980). The maximum concentration of salts in the aqueous phase will be near 10%. All known food-poisoning bacteria can grow at least in the upper part of this range. Staphylococci can grow at one-half of their maximum rate at 0.94 $A_w$ (Scott, 1953) and are likely to be the major concern within this range as evidenced by the fact that lightly salted meat products have been incriminated in staphylococcal food poisoning. At $A_w$ 0.93 or lower, many of the staphylococci competitors, i.e. *Bacillus*, *Clostridium* and *Salmonella* species, grow slowly if at all, making staphylococci the major public health hazard for foods of $A_w$ in this range (Christian, 1980). While enterotoxin production has not been reported in foods below 0.93 $A_w$ (Troller and Stinson, 1975), enterotoxin A formation has been demonstrated at 0.867 $A_w$ (30°C) and 0.887 $A_w$ (25°C) within two weeks in a salt mixture broth (Lotter and Leistner, 1978).

In the physiological studies done by Hughes and Hurst, (1980a), NaCl(5.8%) addition showed a temperature protective
effect on *S. aureus*. The upper temperature limit of growth of *S. aureus* was extended from about 44°C to 46°C. In addition, they observed an increase in heat resistance (D value). The presence of NaCl increased survival of *S. aureus* incubated at higher temperatures and in some foods stored at room temperature. Smith et al. (1982) confirmed that salt protected *S. aureus* from heat injury. Hughes and Hurst (1980b) also described that cell morphology of *S. aureus* grown at 45°C in the presence of 5.8% NaCl differed appreciably from those grown at 37°C in the absence of added NaCl. The septation of the high temperature cells became irregular, they had thickened cell walls and occurred in clumps or clusters, whereas cells grown at 37°C with or without high salt concentration occurred singly. They attributed the increased heat resistance as possibly due both to clumping and the thickened cell walls. Follow up study by Tesone et al. (1981) showed that most food poisoning organisms can be grown above their normal upper limiting temperature in the presence of NaCl, and the protective effect of NaCl applies generally to vegetative cells.

Another factor in the control of microbial spoilage in fermented foods is the production of organic acids, chiefly lactic acid. In their natural state, most foods such as meat, fish and vegetables are slightly acidic, some fruits such as orange and lemon are moderately to highly acidic, and a few foods such as egg white are alkaline. It has been known that the acidity of foods can be increased naturally by fermentation or artificially by
addition of acids to enhance microbiological stability and preserve the foods. Acidity may be the primary factor in preservation or may be an ancillary factor combined with other factors such as chemical preservatives, heat and low water activity. Therefore, it is sometimes difficult to isolate the effect of a single factor. In foods, the acids of interest are almost always weak acids. The change in pH is buffered by the proteins present, thus the acidity may also be important if the organic acids have a bacteriostatic activity dependent on pH. Titratable acidity is the measure of the amount of free hydrogen ions, as well as the amount of hydrogen ions released from undissociated acid during titration. Microorganisms are affected by both the level of free hydrogen ion (pH) and the concentration of undissociated weak acids which is in turn affected by pH. The anions of some weak acids (e.g., acetic or lactic) are metabolized within the bacterial cell, so that H+ is released, acidifying the cell interior to inhibitory levels. Other anions are not so metabolized, therefore do not acidify the cell interior and thus show no inhibitory effect. Many microbes can grow over a wide range of pH values. It used to be assumed that microbial cells possess efficient methods to stabilize their internal pH. However, investigations have shown that internal pH may be considerably affected by the pH of the cell exterior.

Kashket and Wong (1969) measured the flow of a weak acid 5, 5,-dimethyl-2,4-oxazolidinedione (DMO) across the cell membrane of
**E. coli.** In the pH range 5-7 the internal pH of *E. coli* was usually more alkaline than that of the exterior; above pH 7, it was more acid. The effect of a strong inorganic acid (phosphoric) and a weak organic acid (acetic) on the internal acidification of yeast cells was compared by Neal et al. (1965). They found that the weak acid (acetic) acidified the cell interior more effectively than the strong acid. At pH 4, formic, acetic and butyric acids inhibited glycolysis in intact yeast cells. A variety of weak acids at or below their pKa values are potent inhibitors of amino acid transport in *Penicillium chrysogenum*. The effective compounds include sorbate, benzoate and propionate. It has been suggested that the undissociated form of these weak acids may diffuse freely through the cell membrane and ionize in the cell to yield protons that acidify the alkaline cell interior (Hunter and Segel, 1973).

Freese et al. (1973) determined the ratio of pH values inside the microbial cell to those outside, using weak lipophilic acids as preservatives, and concluded that it was the rate of proton leakage into the cell relative to the proton clean-up capacity of the cell that determines the extent to which an environment is inhibitory. Potent preservatives, such as weak lipophilic acids, cause leakage of hydrogen ions across the cell membrane, acidifying the cell interior, and inhibiting nutrient transport. Some acids (e.g., lactate, citrate) will dissociate to give anions which the cell can transport and whose presence does not
therefore inhibit energy-yielding metabolism. Other acids (e.g., acetic, formic) are very effective preservatives since they are not only proton conductors but also may yield inhibitory concentrations of their anion within the cell. Some salts of weak mineral acids (e.g., sulfite, nitrite) of which the degree of dissociation depends on pH become highly inhibitory at low pH.

The pH tolerance of microorganisms thus can be affected by the nature of the acid used for acidification. *Lactobacillus brevis* grew in APT broth to pH 3.0 when acidified with citric, hydrochloric, phosphoric or tartaric acid, to pH 3.7 acidified with lactic acid and to pH 4.0 acidified with acetic acid (Juven, 1976). The acid tolerance of *S. aureus* in pasteurized milk is also altered by the type of acid used to acidify the milk. A 90% reduction in growth rate was observed at pH 5.2 with acetic, at pH 4.9 with lactic, at pH 4.7 with phosphoric or citric and at pH 4.6 with hydrochloric acid (Minor and Marth, 1970). From a study of interactions between staphylococci and saprophytic microbes over a range of pH, Peterson et al. (1964) concluded that the primary effect of pH on staphylococcal growth was direct rather than through its influence on saprophyte growth and competition. The cells of various microbial species have different tolerances toward internal acidification or anion accumulation, and their membranes have different permeability characteristics for lipophilic acids. In a mixed flora, acidity of the food system plays a role in selecting a particularly tolerant component of the
initial population. Low pH foods (e.g., citrus, salad dressing) are found to favor yeasts and lactobacilli (Juven, 1976; Smittle, 1977).

The pH limits for growth vary greatly among microorganisms within the range of about 1-11. In general, yeasts and molds grow at much lower pH than do bacteria, whereas the maximum pH values for growth are similar among bacteria, yeasts and molds. Many microorganisms grow optimally near pH 7 and grow well between pH 5 and 8. Some exceptions are: acetic acid bacteria grow optimally between pH 5.4 and 6.3; lactic acid bacteria grow between pH 5.5 (or below) and 6.0 (Corlett and Brown, 1980). Despite considerable differences in the composition of the cell wall of Gram-positive and Gram-negative organisms, their limits of pH tolerance may show only slight differences. However, the bacteria that survive better in acidic foods (pH < 4.5) are all Gram-positive, and most commonly are species of lactobacilli. In addition to the resistance to lactic or acetic acids, some lactobacilli may produce weak lipophillic acids in sufficient quantities to inhibit enteric bacteria (Cieplinska and Zychowicz, 1974).

From the result of experiments where all conditions other than pH were optimum, the pH range of growth would appear wider than what occurs in foods, when the situation is less than optimum. Other factors, e.g. competing microbial species, unfavorable oxygen tension, adverse storage temperature, reduced
water activity or cell injury could reduce the pH range for growth. Interaction between pH and salt (NaCl) concentration was illustrated as affecting lag phase, reducing growth rate and maximum cell concentration of *S. aureus* (Riemann et al., 1972). The minimum pH permitting growth of *S. aureus* in laboratory medium was 4.5 in 8-10% NaCl and 6.0 in 16% NaCl, and at no pH was toxin synthesized at NaCl concentration above 10%.

When the acidity is sufficiently high to stop microbial growth, the length of time that microbes are able to remain viable becomes critical in assessing their potential health hazard, e.g. food poisoning, should conditions later become favorable for growth. Under adverse conditions, microbes may be deprived of the maintainance energy needed to survive, which leads to loss of viability. When microbes can transport sufficient nutrients for maintainance, even though not enough for growth, they can remain viable for a long time.

A number of food preservation factors may interact with pH to affect the survival of microorganisms. Survival of *S. aureus* (10^8 cells/mL) in trypticase soy broth acidified with acetic, citric, hydrochloric, lactic, and phosphoric acids was investigated by Minor and Marth (1970). Between 90-99.99% of the cells were inactivated within 48 hours when they were exposed to media adjusted to pH 3.6-5.2 with hydrochloric acid. Acetic, lactic and phosphoric were more active against *S. aureus* than were hydrochloric or citric acid. Mixtures of lactic and hydrochloric
acids inactivated a higher proportion of cells than did either acid alone. The killing action was attributed to the undissociated acid molecule, since the anions of the acids had no effect on survival. The survival of *S. aureus* in low water activity foods (0.43 $A_w$) stored for weeks at 25°C was studied by Christian and Stewart (1973). There was a significantly lower survival with decreased pH, which emphasized the importance of the interaction among environmental factors of food system. Although reduced $A_w$ is believed to have major bacteriostatic effect in dried food, other factors such as pH may enhance this effect.

Sodium and potassium salts of nitrate and nitrite are unique ingredients of meat curing mixtures because they serve a variety of functions related to chemical and microbiological properties that influence the microbial safety, quality and identity of cured meat products. Nitrate and nitrite added to meat are converted to an equilibrium mixture of $NO_3^-$, $NO_2^-$ and NO depending on pH and $E_h$ (Shank et al., 1962). The added salts eventually disappear as the result of chemical reactions with the muscle protein, myoglobin, to form the characteristic cured meat color (Draudt and Deatherage, 1956; Fox, 1966; Bard and Townsend, 1971; Cassens et al., 1979). The reactions may involve the heme pigments (Mohler, 1973) and the nonheme protein (Woolford et al., 1976). Nitrite is also associated with the flavor of cured meats and it serves as a mild antioxidant preventing off-flavors and odors (Cho and Bratzler, 1970; Wasserman and Talley, 1972; Hustad et al., 1973;
The very important function of nitrite concerning food safety is its role in delaying *Clostridium botulinum* growth and toxin production when cured meat products are abused at elevated temperatures. Reddy et al. (1983) used the high resolution electron spin resonance method to demonstrate that added nitrite reacted with iron-sulfur proteins present in vegetative cells of *C. botulinum* to form iron-nitric oxide complexes, resulting in destruction of the iron-sulfur cluster. Inactivation of iron-sulfur enzymes (especially ferredoxin), whose metabolic roles in anaerobic bacteria include electron transport and generation of adenosine triphosphate via binding of nitric oxide, would almost certainly halt growth of *C. botulinum*. The effects of heme-iron binding gases (NO and CO) and of iron-chelating agents on growth and toxin production by a mixed inoculum of *C. botulinum* types A and B in pork slurries were studied by Vahabzadeh et al., 1983. They revealed that nitric oxide had similar effects to that of sodium nitrite and residual nitrite was present in the NO-treated meat. Addition of ferric chloride or myoglobin decreased the antibotulinal effectiveness of 156 ppm sodium nitrite, but not when ethylene-diaminetetraacetate (EDTA) or denatured nitrosylated myoglobin was also present. They concluded that supplemental iron compounds probably decreased residual nitrite level in the product, thus permitting botulinal growth, rather than directly stimulating *C. botulinum* growth by providing iron as an external
nutrient. The antimicrobial activity of nitrite depends on pH (Tarr, 1941a, 1941b, 1942, 1944; Castellani and Niven, 1955; Shank et al., 1962; Roberts and Ingram, 1973; Riha and Solberg, 1975a, 1975b) and increases as the pH decreases from 7.0 to 5.0. The inhibitory activity of nitrite equates well with concentration of undissociated nitrous acid (HNO₂); 3500 ppm of NaN0₂ at pH 6.9 and 40 ppm at pH 5.0 yield almost identical concentrations (about 1 ppm) of undissociated HNO₂ (Castellani and Niven, 1955). Redox potential may also affect the antimicrobial activity of nitrite against some but not all species (Castellani and Niven, 1955).

The rate of disappearance of residual nitrite in cured meat products depends on pH and temperature; as the pH drops and the temperature rises, the rate of disappearance becomes more rapid (Olsman and Krol, 1972). Some microbes may also cause the lowering of residual nitrite by using it as a hydrogen acceptor. The growth of some nitrite-reducing bacteria persists until the nitrite is exhausted. Bacterial reduction of nitrate or nitrite to N₂O and N₂ may cause swelling in cured meat products (Eddy and Ingram, 1956).

At the level commercially used, nitrite does not cause rapid destruction of undesirable microorganisms. Rather it retards or prevents their growth. In the case of C. botulinum, nitrite does not prevent spore germination but prevents outgrowth (Gould, 1964; Duncan and Foster, 1968; Pivnick et al., 1970). Depending on the concentration of nitrite, germinated spores and vegetative cells
may grow, or they may die slowly (Pivnick et al., 1970; Christiansen et al., 1978; Tompkin et al., 1978c). If growth occurs, it may be uninhibited or it may be decreased as the concentration of nitrite is increased (Riha and Solberg, 1975b). The rate of death is a function of pH and concentration of nitrite (Tarr, 1942). At pH 4.0-5.5, rapid destruction of vegetative cells and probably spores of some species may occur (Shank et al., 1962).

Owing to the formation of nitrosamines which resulted from the addition of nitrate and nitrite to foods, various studies have been done to develop procedures aimed at the elimination or lowering of levels of nitrate and nitrite required for growth inhibition of \textit{C. botulinum}. Studies have shown that it is possible to lower nitrite levels (40 μ g per g) when used in combination with sorbic acid or potassium sorbate in bacon and canned comminuted pork (Ivey et al., 1978; Ivey and Robach, 1978; Robach et al., 1978). With nitrite alone, the effect of sorbic acid and nitrite for the control of \textit{C. botulinum} is also pH dependent. The inhibitory effect is not observed at pH values above 6.2 and the effectiveness increases with decreasing pH (Sofos et al., 1980). The effectiveness of combinations of sorbic acid and nitrite in preventing outgrowth of \textit{C. sporogenes} has also been demonstrated (Robach, 1979). Besides controlling toxin production and lowering levels of nitrosamine, these combinations have the advantage of retaining much of the traditional identity of the products (Ivey et al., 1978; Paquette et al., 1980; Robach
et al., 1980). Other compounds found to enhance the inhibitory effect of nitrite are ascorbate and/or isoascorbate. They were originally used in cured meat products mainly as accelerators of the curing reaction. They also contribute to the development of more stable color and act as synergists to antioxidants. Ascorbate is also believed to decrease the rate of nitrosamine formation either by increasing the rate of residual nitrite depletion (Bowen and Deibel, 1974; Woolford and Cassens, 1977) or directly blocking the nitrosation of amines by nitrite (Mirvish et al., 1972). Tompkin et al. (1978) reported that addition for each gram of meat of 50 μg NaNO₂, together with 0.02% sodium isoascorbate, was as effective as 156 μg of NaNO₂ alone in delaying swelling of cans containing comminuted cured pork inoculated with C. botulinum spores.

Numerous studies have been done on the interaction of salt (NaCl), nitrite, temperature, pH, Eₜ, Aₓ, fermentation and other factors to assess safety against several pathogens and spoilage organisms in many foods. Formulae for predicting the number of S. aureus required to initiate growth with inhibiting agents, pH and salt interacting in laboratory media have been developed. The conclusion was that the number of cells required to initiate growth in meat under the same conditions of pH and salt is considerably less than in media (Genigeorgis et al., 1971a, 1971b). The complexity of these interactions makes it difficult to predict whether or not bacterial safety of the products will be
maintained when one or more factors are changed.
CHAPTER III

SURVIVAL OF TRICHINELLA SPIRALIS IN NHAM

(THAI-STYLE FERMENTED PORK SAUSAGE)

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Abstract

Lean ground pork pooled from 2 pigs experimentally infected with *Trichinella spiralis* was divided into 4 portions. Three portions were frozen at -23°C for 12, 18 and 24 hours before being made into Nham (Thai-style fermented pork sausage). The formula of Nham included 3% w/w NaCl and garlic, 0.015% each of NaNO₂ and NaNO₃, and either 1.0% or 1.5% commercial starter culture. Fermentation without starter culture resulted in Nham that induced parasitism in rats. Infected pork, frozen for 24 hours, was still found to cause a low level of parasitism. However, the corresponding fermented sausages were not found to be infective. Use of a lower level of starter culture (1.0%) and a freezing time of 12 hours rendered trichinae larvae noninfective after 2 days of fermentation. The higher level of starter culture caused faster acid production at 30°C but no difference in the maximum number of lactic acid bacteria and final pH attained. If fermentation is to be relied upon for the safety of Nham, the addition of the higher level (1.5%) of starter culture (Nham powder) appeared to be an alternative to freezing.
Introduction

Fermented pork sausage (Nham) is a very popular food in Thailand even though there is a possibility of the product causing trichinosis in the consumer because the pork is not heat-processed. An outbreak of trichinosis which occurred in Chiang Mai Province in 1975 was traced to Nham (Khamboonruang et al., 1978). In the latest survey of the epidemiology of trichinosis in Thailand in 1980, the hill tribe pig was shown to be the major reservoir host for human infections (Dissamarn et al., 1980).

Control of infective trichinae in western-style processed meats has been investigated in the U.S. (Gammon et al., 1968; Crouse and Kemp, 1969; Zimmermann, 1971; Terrell et al., 1981; Kayfus et al., 1982; Childers et al., 1982). In the study of the effect of NaCl alone (Allen and Goldberg, 1962), trichinae larvae survived 3.5% w/w salt for 31-60 days in ground meat, and for 49-79 days with 2.5% w/w salt. Preprocessing treatment, such as freezing using selected time-temperature combinations recommended by the U.S.D.A., is one approach for control of trichinosis. In a recent study to evaluate the relationship of storage time and temperature for the loss of infectivity of Trichinella spiralis under simulated shipping conditions, 6-10 days at \(-17.8^\circ C\) were required to rendered the parasites noninfective (Zimmermann et al., 1985).

Freezing may be a widely applied method for the control of trichinae in pork to be used in raw sausages in the U.S. but...
because it is the most expensive in view of energy costs, its use in a developing country such as Thailand is limited. So far, the control of this parasite has relied on fermentation and the recommendation of cooking before consumption. The success of the latter is in question due to the conflict with ethnic groups' preferences.

The objectives of this study were to determine the effects of different levels of starter culture and/or freezing on viability of *T. spiralis* in Thai fermented pork sausage, and to identify the combination of factors which would assure destruction of *T. spiralis* infective larvae.

**Materials and Methods**

Two pigs (90-100 kg) infected with 800 larvae per g of weanling weight (15 kg) were used to prepare sausages. Pooled, lean muscles (containing 1-52 larvae per g) were stored at 3°C and processed within 1 wk. Triplicate samples were allocated to the following treatment combinations: (a) starter culture levels of 1.0% and 1.5% w/w; (b) frozen at -23°C for 12 hr, and 1.0% or 1.5% w/w starter culture; (c) frozen at -23°C for 18 hr, and 1.0% or 1.5% w/w starter culture; (d) frozen at -23°C for 24 hr, and 1.0% or 1.5% w/w starter culture.

Controls (no starter culture) were made for each treatment to determine the base level of infectivity. Sausages were made using the basic formula which included 3% w/w NaCl, 3% w/w chopped
garlic and 0.015% w/w each of NaNO₂ and NaNO₃. Starter culture packets (Nham Powder) prepared by Griffith Laboratories Ltd., Thailand, contained dried culture, delta gluconolactone, dextrose, sodium phosphate and sodium erythorbate. Mixing was done in batches with a Kitchen Aid Mixer (Model 3-C, Hobart Manufacturing Co., Troy, Ohio). In an attempt to simulate a typical small scale production, the meat was reground with chopped garlic before the appropriate quantities of NaNO₂, NaNO₃ and NaCl, which were thoroughly mixed, were added. Mixing was done at medium speed for 2 min before the starter culture was added and again thoroughly mixed. The mixture was divided into 50 g portions and each placed in a half-pint Ziplock plastic bag. The sausage was tightly rolled to eliminate air as much as possible before the bag was closed and incubated at 30°C. Sausages were removed from the incubator at 24 hr intervals from 0 to 4 days of fermentation and analyzed for trichinae infectivity, total aerobic plate count, total lactic acid bacteria count, pH and total acidity.

The infectivity of *T. spiralis* in the sausage was determined using rat bioassays. The encysted larvae were first extracted from sausages using the artificial digestion procedure (Zimmermann and Hubbard, 1969) as modified by Childers et al., 1982. Two mL of the final slurry were then fed to 2 rats (1 mL each) via a stomach-feed needle. After 40 days, the rats were sacrificed and quantified for *T. spiralis* encysted larvae using the same extrac-
tion method. Three mL of the final slurry were transferred into a sectioned counting chamber and viewed microscopically.

Serial dilutions were prepared from a 20 g representative sample of sausage from each bag in 0.1% peptone water. Total aerobic plate counts were enumerated with Plate Count Agar (Difco) and lactic acid bacteria with MRS medium (Difco). These were done by the spread-plate technique using 0.1 mL of appropriate dilutions. All plates were incubated at 30°C for 48 hr before colony counts were recorded.

The pH of sausages was measured with a Corning pH meter Model 7 and a combination glass electrode (Orion Research Inc., Cambridge, MA), (A.O.A.C., 32.010, 1984). The total titratable acid as expressed as percent lactic acid was also determined (A.O.A.C., 16.276, 1984). This experiment was a split plot design of two complete replications with data tested by analysis of variance. Part of the data was analyzed by use of paired t-distribution. The significance level chosen was 0.05.

Results and Discussion

Fermentation was rapid during the first four days in all treatments. In the control treatment, the pH value (Figure 3.1) at the beginning of fermentation was 5.4 and the total acidity as % lactic acid (Figure 3.2), was about 1.3%. The sharpest drop in pH value occurred during the first 24 hr in all treatments. On the fourth day, the control product was considered to have
attained typical organoleptic characteristics; the minimum pH reached was 4.8 and the acidity was 1.6%. The addition of starter culture resulted in lowering the initial pH of the composite by the acidulating action of delta-gluconolactone; however, the rate of fermentation was not significantly different (P>0.05). The minimum pH attained in the 1.0% and 1.5% starter culture was 4.7 and 4.6 and the maximum titratable acidity was 1.8% and 1.9% lactic acid, respectively.

Bacterial counts on standard plate count agar and MRS agar were similar in all samples (Figure 3.3). They began in the range of 1.3x10^6 CFU per g and ended in the range of 1.0-1.8x10^8 CFU per g. Addition of either level of starter culture resulted in shortening of the time required to reach maximum counts from 3-4 days to 1-2 days (P>0.05). Differential counts of lactic acid bacteria on MRS agar showed a sharp shift in the type of microorganisms present. During the early stage, the Gram-positive to Gram-variable coccobacilli predominated, then disappeared concomitantly with the progressive increase in number of Gram-positive rods which then persisted until the later stage. In the control treatment, this shift was gradual and still ongoing at the end of the four days fermentation. With the addition of starter culture, the process was completed within 48 hr (1.0%) and 24 hr (1.5%).

Bioassay results of infectivity of _T. spiralis_ larvae from fermented meat are shown in Table 3.1. The number of viable
larvae in rats fed with sausage extract (not frozen, not fermented) ranged from nondetectable to 2229 larvae per g (LPG) despite an attempt to thoroughly mix the meat. This high variation may have been due to the low level of infection in one of the two pigs used and the varied level of LPG in different tissues within the same host. The non-random distribution of encysted larvae among tissues is not an unusual phenomenon in pigs of approximately the same age and weight inoculated with the same inoculum (Kotula et al., 1984). Fermentation without starter culture did not show any effect on infectivity; larvae present ranged from 106 to 881 LPG in the second day and 302 to 573 LPG in the fourth day. With 1.0% starter culture, *T. spiralis* survived through the fourth day but caused only a low level of infection in rats. Infective larvae were not detectable after the second day when 1.5% starter culture was added.

After being frozen at -23°C for 12 hr, *T. spiralis* was still capable of causing parasitism ranging from low (45 LPG) to moderate (400 LPG) levels. After 18 hr, the meat caused only a very low level of infection in rats of replication 1 but not in replication 2. After 24 hr, there was only one rat that had a very low level of infection. Interaction of levels of starter culture and freezing time was significant (P<0.05) after 12 hr.

The effectiveness of salting as the means for destroying trichinae in pork products has been evaluated and approved to use in conjunction with other methods such as freezing or heating.
(U.S.D.A., 1973). In the study of Allen and Goldberg (1962) of the effect of salt alone, trichinae larvae were found to survive 2.5% to 3.5% w/w NaCl for up to 60-76 days, respectively. With the level of 3.0% NaCl, 0.015% nitrite and nitrate salts and the fermentation time of 4 days used in all treatments, no influence was exerted upon the infectivity of *T. spiralis* larvae as evident by their persistence in the control treatment throughout. The level of NaCl chosen was that recommended by Yanasugondha (1977) for commercial and household production that guaranteed successful and organoleptically acceptable sausage. Addition of starter culture at either level resulted in a more acidic environment and affected the infectivity of trichinae.

**Summary**

The use of starter culture shortened the time required for natural fermentation from 3-4 days to 1-2 days but the final pH attained was not affected by the higher level of starter culture. The rate of shift in the major groups of bacteria involved in fermentation was greatly affected by the level of starter culture. However, the total plate counts and lactic acid bacteria counts were comparable. Fermentation of Nham without added starter culture did not assure the complete destruction of *T. spiralis* larvae; as expected, combining it with prefreezing of the pork was effective. However, infected pork, after being frozen at -23°C for 24 hr, still caused a low level parasitism in
one of the tested rats. In fermented sausages, corresponding samples gave negative results. There was significantly greater survival of trichinae larvae in sausages made from meat frozen for 12 hr and fermented without starter culture. If fermentation is to be relied upon for the safety of Nham, the higher level (1.5%) of Nham powder is suggested to be the alternative to freezing.
Figure 3.1. Changes in pH during the fermentation of Nham (Thai-style fermented pork sausage) made with and without starter culture.
Figure 3.2. Changes in titratable acidity during the fermentation of Nham (Thai-style fermented pork sausage) made with and without starter culture.
Figure 3.3. Changes in numbers of lactic acid bacteria and total plate counts during the fermentation of Nham (Thai-style fermented pork sausage) made with and without starter.
Table 3.1. Bioassay results of infectivity of Trichinella spiralis larvae from control pork and pork frozen and thawed, made into Nham (Thai-style fermented pork sausage).

<table>
<thead>
<tr>
<th>Frozen Time a,b (hr)</th>
<th>Starter Culture Level c (% w/w)</th>
<th>Rep</th>
<th>Viable trichinae per gram d</th>
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<tr>
<td></td>
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<td>Fermentation time (days)</td>
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<td>2</td>
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</tr>
</tbody>
</table>

a Freeze 1 kg of meat in polyethylene freezer bag (27 x 30 x 2 cm³) at -23°C.

b Submerge in cold running tap water.

c Nham Powder: Griffith Laboratories Ltd., Thailand.

d Average of two duplicate rat bioassays unless indicated otherwise.

e Not detectable.

f One sample was negative.
REFERENCES


CHAPTER IV

FATE OF

STAPHYLOCOCCUS AUREUS AND ESCHERICHIA COLI

IN NHAM (THAI-STYLE FERMENTED PORK SAUSAGE)

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Submitted to the Journal of Food Protection
Abstract

The fate of *Staphylococcus aureus* and *Escherichia coli* was determined when they were introduced into ground pork made into Nham (Thai-style fermented pork sausage) with or without 0.75 or 1.5% added starter culture. Without starter culture, the numbers of *E. coli* remained with little change but there was slow multiplication of *S. aureus*. With 0.75% starter culture, *S. aureus* was no longer detectable after 48 hours and *E. coli* numbers decreased by 1 log after 96 hours. No viable *S. aureus* or *E. coli* were recovered after 36 hours and 96 hours, respectively, when 1.5% starter culture was added. The addition of a starter culture is recommended when making Nham, a Thai fermented sausage.
Introduction

Fermented pork sausage (Nham) is a very popular food in Thailand. It consists of fresh lean ground pork, mixed thoroughly with salt and garlic and traditionally wrapped in small banana leaf packets (34). After 3-4 days at room temperature, a natural lactic acid fermentation occurs and the sausage develops an acidic flavor similar to western sausages fermented with pediococci. Nham is then generally considered ready for consumption. Nham should be used as soon as it reached the final pH (4.6±0.1). The texture should be firm when pressed between the fingers. At this early stage, it would remain in good condition for another 24 to 48 hr without refrigeration. If it is not going to be consumed immediately, it should be refrigerated but kept no longer than 2 weeks. If it is cooked before serving, as now recommended, no problem would be involved. But in general, this fermented sausage is still consumed without any further heat treatment, thus the question of the viability of microorganisms that can cause food-borne illness is of public health significance.

*Staphylococcus aureus* and *Escherichia coli* are frequently found in raw meat as the result of human handling, meat handling surface and the skin or hides of animals during processing (2,13,25,28,29,32). Careless meat handling techniques during the production of fermented sausage can lead to staphylococcal development and outbreaks of illness. Given the proper conditions, *S. aureus* can multiply and produce toxin during the initial hours of
fermentation. In 1971, several outbreaks of food poisoning were traced to Genoa salami containing up to $10^8$ CFU per g of *S. aureus* and type A enterotoxin was detected in some of these samples (30,31). In the simulated study of enterotoxin production in Genoa salami (15), staphylococcal populations of $10^7$ to $5 \times 10^8$ CFU per g were concurrently detected with measurable amounts of enterotoxin A. Although the production of enterotoxin is always dependent upon attainment of high cell populations, the presence of enterotoxin can sometimes occur with no viable cells of *S. aureus* in corresponding samples due to prior cell death (3,21,23). Ingestion of the thermostable preformed enterotoxins results in acute (1-8 hr) symptoms which include diarrhea, vomiting, nausea, abdominal cramps and prostration. Most patients recover within 24-48 hr and death is rare (8). Use of starter cultures of lactic acid bacteria has facilitated inhibition of staphylococcal multiplication during processing of sausage (4). However, "chance inoculation" is still commonly used to some extent, especially in a household setting.

Enterotoxigenic *E. coli* (ETEC) is a common cause of diarrheal disease in swine and human (11,17,22,26). The transmissible plasmid-mediated nature of *E. coli* host specific adherence factor (14), human-specific gastrointestinal colonization factor (6) and production of enterotoxin by some strains (12), has created increasing concern for the presence of *E. coli* in foods as agents of foodborne illness. In a survey of the prevalence of ETEC in
Thailand, ETEC was found to be wide spread in suckling pigs with diarrhea, water sources on pig farms and pig handlers with or without any gastrointestinal symptoms (5). The lactic acid fermentation controls growth of *E. coli* mainly through lowering of pH and antibiotics and other growth inhibitor production (19). In fermented dairy products, survival of *E. coli* varies, depending on starter culture, pH, temperature of storage and composition of the product (10, 19, 20, 33). The lactic acid fermentation does not necessarily guarantee the safety of the products (7, 16).

The purpose of this study was to determine the multiplication and/or survival of *S. aureus* and *E. coli* when in competition with the lactic acid fermentative starter culture of Nham, using initial population and a growth temperature which could occur during a typical small scale production.

**Materials and Methods**

**Sausage Preparation**

Lean pork trimmed of fat from two hogs, slaughtered in the university facilities, was pooled, divided into 1 Kg portions, packaged in polyethylene freezer bags and frozen (-23°C) until used. Meat was taken from the freezer and placed in a cold room (3°C) 1 day prior to sausage preparation. Partially thawed meat was ground in a commercial meat grinder. Ground meat was mixed thoroughly and divided into 3 equal portions (1 kg): a control, a
culture. The starter culture which was obtained from a local Asian grocery store, had been prepared by Griffith Laboratories Ltd., Thailand, and contained dried culture, delta-gluconolactone, dextrose, sodium phosphate and sodium erythorbate. The other ingredients in the sausage formula were 3% w/w chopped garlic and NaCl, 0.015% w/w each of NaNO₂ and NaNO₃. The NaCl, NaNO₂ and NaNO₃ were mixed together before being sprinkled over the ground meat. After mixing at medium speed with a Kitchen Aid Mixer (Hobart Manufacturing Co., Troy, OH) for 2 min, starter culture and garlic were distributed over the meat mixture and mixed at medium speed for another 2 min. Each batch was divided into 40 g portions and each portion placed in a half-pint storage Ziplock bag. A mixed inoculum of \textit{S. aureus} and \textit{E. coli} in 1.2 mL of predetermined suspension was added to each of half of the sausages. Each sausage was tightly rolled to eliminate as much air as possible before the bag was closed and incubated at 30°C. Sampling intervals were 0, 2 hr between 5 and 13 hr, 6 hr between 13 and 24 hr and 12 hr afterward through 96 hr.

**Microbial Inoculation**

\textit{S. aureus} strains FRI 100, 1000 and 1004 were obtained from the stock cultures of the Food Research Institute (FRI) at the University of Wisconsin-Madison. FRI 100, an enterotoxin A producer (SEA), is the classical strain originally isolated in 1932 by E.O. Jordan of the University of Chicago, from a cake impli-
cated in a food poisoning outbreak. FRI 1000 (SEA) and FRI 1004 (SEC) were isolated in 1979 at the FRI from a Genoa salami. *E. coli* strains 2C, 185 and 198 were from the culture collection of the Department of Microbiology, Oregon State University. All three showed typical coliform type I characteristics. All stock cultures were maintained on Trypticase Soy Agar (TSA, Difco) and transferred onto TSA slants and incubated at 30°C for 24 hr before use.

Preliminary assays were conducted to quantitate the appropriate level of inoculum of *S. aureus* and *E. coli*. Cells were washed from the slant and adjusted to a transmittance of 65% at 620 nm with sterile 1% peptone water just prior to inoculation. A sterile pasteur pipette was used to distribute 1.2 mL of mixed inoculum to 40 g sausage to give initial concentrations of $10^3$ CFU per g of *S. aureus* and $10^4$ CFU per g of *E. coli*.

**Microbial Determinations**

A 25 g representative sample was taken from each bag aseptically and added to 225 mL of sterile 0.1% peptone water in a sterile Stomacher bag. Homogenizing was done in a Stomacher 400 (Tekmar Company, Cincinnati, OH) for 2 min. Serial dilutions were then made in 0.1% peptone water. Aerobic plate counts were enumerated with Plate Count Agar (Difco) and lactic acid bacteria with MRS Agar (Difco). *S. aureus* was determined with Baird-Parker medium with 5 % added egg yolk (BBL and Difco). These were done by the spread-plate technique using 0.1 mL aliquots. For *E. coli*.
Violet Red Bile Agar (Difco) pour-plates, overlaid with 5 mL VRBA, were used. To differentiate lactic acid bacteria (24), isolated colonies from MRS plates were tested for Gram-stain, cell morphology, motility, spore formation, catalase production, hydrolysis of gelatin/arginine, utilization of sugars, nitrate reduction and IMViC reactions. All plates were incubated at 30°C for 48 hr.

**pH and Total Acidity**

The pH of sausage samples was determined (A.O.A.C. 32.010, 1984). Total acidity, expressed as % lactic acid, was also determined (A.O.A.C. 16.276, 1984).

**Experimental Design**

Three trials were done for each level of starter culture. Effect of factors included was determined by ANOVA. The level of significance was established at 5%.

**Results and Discussion**

**Fermentation**

Under the fermentation conditions recommended (34), reduction in pH occurred after 12 hr (Figure 4.1) and production of lactic acid was steady and rapid for the first 48 hr. By 60 hr, the pH had gradually decreased to 4.8±0.1. The presence of *S. aureus* and
*E. coli* did not change the rate of acid production. The addition of 1.5% starter culture resulted in a decrease in the initial pH and a more immediate fall in pH values, indicating that there was no delay in the onset of fermentation. With 0.75% starter culture, the initial pH was only slightly lower than the control but fermentation also began immediately. The lowering of the initial pH was due to the presence of delta-gluconolactone. The different levels of starter culture resulted in significantly different rates of acid production ($F=106.33$, $P<0.05$) and decrease in pH ($F=105.36$, $P=0.05$). After 72 hr, when the products were considered to have attained the flavor and texture characteristics of being ready for consumption, the minimum pH reached ($4.8\pm0.1$) of control treatments were still higher than the recommended value ($4.6\pm0.1$). With 0.75% and 1.5% starter culture, pH after 72 hr was 4.7.

The sausage containing 1.5% starter culture had reached the recommended pH after an average of 48 hr. Sausages with 0.75% starter culture reached that same pH in approximately 60 hr. Without the addition of starter culture, fermentation onset was delayed for 12 hr and the final pH plateaued at 4.8 after 72 hr.

**Microbial Populations**

Bacterial counts on PCA and MRS were similar in all treatments after 24 hr. The maximum numbers of CFU reached were also comparable (Figure 4.2) within the range of $4.0-5.0\times10^8$ CFU per g.
The differential counts of lactic acid bacteria presented as a percentage of the total microbial population (Figure 4.3) indicated that two major groups of bacteria prevailed at different stages of fermentation. During the early stage, pediococci predominated. They were Gram-positive to Gram-variable cocci, clustered or in pairs and rarely single, cell diameter approximately 0.4-0.6 μm, catalase-negative, negative for nitrate reduction, non-motile and non-sporeforming. Colonies were raised and circular (1 mm in diameter) on MRS but pinpoint on PCA with filiform growth on stab culture. All isolates could ferment sucrose but the ability to utilize arabinose varied. This group gradually decreased and the second group came to prominence after 48 hr. These lactobacilli consisted of Gram-positive rods, non-sporeforming, catalase-negative, negative for nitrate reduction, non-motile, appeared in chains and singly with cell size of 1-1.5×6 μm, and white, raised and circular colonies (1-2 mm in diameter) on MRS agar but smaller (<1 mm) on PCA. All isolates could utilize melibiose but not rhamnose. The ability to utilize sorbitol varied. The rate of change between the two groups depended on the presence and quantity of starter culture. In the control treatment, pediococci did not completely disappear until after 72 hr. Addition of starter culture reduced this time to 30 hr for 1.5% and 48 hr for 0.75%. The higher level (1.5%) also produced the higher percentage in the initial level of lactobacilli, as expected.

**Staphylococcus aureus**
Numbers of \textit{S. aureus} are shown in Figure 4.4. No \textit{S. aureus} were isolated from the raw meat. When $10^3$ cells per g of \textit{S. aureus} were introduced, they were able to multiply to a limited extent during the fermentation period. The level reached after 4 days was $4.2 \times 10^4$ CFU per g. The staphyloccocal multiplication was controlled by the addition of 0.75% and 1.5% starter culture. Numbers showed a steady decrease and reached undetectable levels at approximately 48 hr and 30 hr, respectively. The initial increase in the 0.75% treatment was not statistically significant ($P>0.05$). The more rapid acid production, which resulted from the addition of 1.5% starter culture, prevented \textit{S. aureus} from increasing in number and also exerted a killing effect as seen by the one logarithm drop after 30 hr. A minimum level of $10^6$ cells of \textit{S. aureus} per g is generally considered necessary to implicate the product in foodborne illness (18,30,31) and more than $4 \times 10^7$ cells per g for detectable enterotoxin production.

Several previous workers showed that \textit{S. aureus} does not grow well in a fermented sausage environment at temperatures lower than 25°C and pH lower than 5.0 (3,15,27), even though meat environments were more conducive to growth than were laboratory media. Lactic acid produced during fermentation caused injury to \textit{S. aureus} (27) and this could be enhanced by the addition of starter culture. Smith and Palumbo (27) concluded that both glucose and starter culture were necessary to produce injury to \textit{S. aureus} during
sausage manufacturing, and the higher the glucose concentration, the faster the rate of killing. At higher glucose levels, the starter culture produced larger amounts of lactic acid. In the commercial starter culture preparation used, dextrose was listed among minor ingredients. Sausages such as Nham contain low levels of fermentable carbohydrates which may be less than sufficient to produce a low final pH. During the first 3 days when acid production is not enough to damage S. aureus, viable cells may multiply in the sausage. This poses a hazard since the numbers of S. aureus were still increasing at the end of the test period. The level of starter culture used was not critical in preventing the multiplication of S. aureus. With the addition of 0.75% starter culture, a slower rate of acid production during the first 12 hr allowed a slight multiplication of S. aureus before cells died.

*Escherichia coli*

The data for *E. coli* from the same inoculated sausages are shown in Figure 4.5. *E. coli* was also susceptible to inhibition by faster starter culture multiplication and acid production. The level of naturally present *E. coli* in this ground meat was low. Inoculated sausages with *E. coli* showed similar early multiplication followed by a subsequent decline in numbers. Adding starter culture resulted in decreasing numbers after 12 hr for both levels. After 96 hr, *E. coli* disappeared completely from the 1.5% sample but survived at low levels ($10^2$ cells per g) in the 0.75%
sample. Without starter culture, the fermentation process exerted only a repressive effect which stabilized the number of viable E. coli.

The final pH of all treatments ranged from 4.6 to 4.9. Control sausages were the only samples whose pH was not close to the recommended pH (4.6±0.1 (34)) after 4 days incubation. Longer survival of these strains occurred because of the lower level of acid produced.

Inhibiting factors

The initial reduction of pH, resulting from added starter culture, caused a marked effect on growth of S. aureus. Barber and Deibel (3) reported a similar effect when they indicated that growth of S. aureus could be controlled with 1.5% delta-gluconolactone, whereas a high inoculum of Pediococcus cerevisiae failed to suppress aerobic growth. Control of S. aureus in fermented dairy products by lactic cultures and chemical acidulation was also reported by Daly et al.(4). It is apparent that acid production is not the sole cause in the reduction of S. aureus and E. coli in sausages. When the starter culture was added (0.75% or 1.5%), the killing effect was more pronounced than the pH differences. This antagonism of lactic acid bacteria toward the introduced pathogens possibly resulted from the production of antibiotics, hydrogen peroxide, volatile fatty acids and acid other than lactic (9).
Summary

This investigation was done to determine the response of *S. aureus* and *E. coli* when in competition with the natural flora or lactic acid bacteria starter culture in the making of Nham. Adding starter culture at either a lower or higher level increased the rate of acid production resulting in a faster drop in pH and a decline in numbers of both *S. aureus* and *E. coli*. Fermentation without starter culture stabilized the number of *E. coli*, but permitted slow multiplication of *S. aureus*. Even though the number of *S. aureus* reached at the time the fermentation was terminated was still lower than what is considered to be the minimum number required for enterotoxin to be detected, the safety of these sausages would be in question if they remained at temperatures which would permit further multiplication. The data presented indicate that natural fermentation of Nham cannot guarantee the complete destruction of foodborne pathogens if they were present. Use of starter is recommended. The level used must be compatible with other factors and not adversely affect the organoleptic quality of the finished products.
Figure 4.1. Changes in pH and titratable acidity during the fermentation of Nham (Thai-style fermented pork sausage).
Figure 4.2. Changes in numbers of lactic acid bacteria and total plate counts during the fermentation of Nham (Thai-style fermented pork sausage)
Figure 4.3. Changes in the percentage of Lactobacilli and Pediococci during the fermentation of Nham (Thai-style fermented pork sausage).
Figure 4.4 Changes in numbers of Staphylococcus aureus when introduced into Nham (Thai-style fermented pork sausage) made with and without added starter culture.
Figure 4.5. Changes in numbers of *Escherichia coli* when introduced into Nham (Thai-style fermented pork sausage) made with and without added starter culture.
REFERENCES


yogurt, buttermilk, sour cream, and cottage cheese during refrigerated storage. Journal of Milk and Food Technology. 34:54-58.


CHAPTER V

ESCHERICHIA COLI ENUMERATION IN THAI-STYLE FERMENTED PORK SAUSAGES WITH DIRECT PLATING AND MOST PROBABLE NUMBER TECHNIQUES

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Abstract

The rapid direct plating technique (RPT) of *Escherichia coli* in foods (direct plating onto cellulose acetate membranes overlaying tryptone bile agar) was compared to two generally used methods: (a) colony counts on violet red bile agar (VRBA) and (b) MPN determination with lauryl sulfate tryptose broth (LST) and brilliant green lactose bile broth (BGLB). Total coliforms from 43 Thai-fermented pork sausages were enumerated. The RPT gave data highly correlated to those from VRBA but with a lower correlation to MPN. The regression line of log_{10} coliform counts from the RPT and VRBA had a slope of 0.76 and an intercept of 0.98. The MPN assay gave significantly higher (P<0.05) numbers than either the VRBA or the RPT.
The detection and enumeration of indicator bacteria are of primary importance for monitoring sanitation and microbiological quality of food and water. *E. coli* is often used as an indicator because it is specific and most reliably reflects fecal origin. In the United States, the Most Probable Number (MPN) assay and the direct plating procedure with Violet Red Bile Agar (VRBA) are accepted for total coliforms of food and dairy products analysis (1). These tests are laborious, costly and time-consuming; MPN requires 96 hr to complete. This concern has prompted the development of a number of rapid methods for enumeration of *E. coli*, among which is the direct plating onto cellulose acetate membranes overlaying tryptone bile agar (RPT)(2). This method was selected by the International Commission on Microbiological Specifications for Foods (ICMSF) as the most promising. It yields results in 24-40 hr, eliminates the use of costly multiple tubes of media, and reduces the requirement for analysis time. A review of this method was done on frozen and non-frozen raw meats (5). This note reports the results of enumeration of *E. coli* in raw fermented pork sausages using the Anderson and Baird-Parker method (2) as compared to VRBA and MPN assay.

**Materials and Methods**

Sausages were prepared in batches from pork trimmed of fat. The formulae included 3% w/w of NaCl and chopped garlic, 0.015% each of NaNO₂ and NaNO₃ and 0.75% or 1.5% starter culture (Nham
Powder, Griffith Laboratories Ltd., Thailand). After mixing, the sausage was divided into 40 g portions, each of which was placed in a half-pint Zip Lock plastic bag. A 1.2 mL mixture of 3 strains of typical *E. coli* (IMViC++) was added and mixed thoroughly to give $10^4$ CFU per g sausage. The sausage was tightly rolled to eliminate as much air as possible before the bag was closed and incubated at 30°C for 4 days as is usually done. Sausages were pulled for microbiological analysis at 2 hr intervals between 5 and 13 hr, 6 hr intervals through 24 hr and then 12 hr intervals. A total of 43 samples were analyzed.

Initial 1:10 dilutions were prepared using 0.1% peptone water and blended in a Stomacher 400 Lab Blender (Tekmar Co., Cincinnati, Ohio) for 2 min. The standard 3-tubes MPN using LST broth and BGLB broth (Difco) (46.016,(3)), direct plating on VRBA (Difco) (1) and direct plating onto cellulose acetate membranes type GN-6 (Gelman Science Inc., Ann Arbor, Michigan) overlaying tryptone bile agar (2) were followed for the total coliform analysis of each sample. The comparison was done using paired t-test and correlation analyses. MPN results which exceeded 2400 MPN index per g were not used in the analysis.

**Results and Discussion**

The correlations among the three groups were examined using bivariate statistic of log10 coliform counts from one method and the second or the third method. The statistical analyses are
presented in Table 5.1. Data from the rapid method (RPT) and VRBA showed the highest correlation coefficient (0.94) and the strongest linear relationship when they were regressed with 95% confidence level. The paired t-test indicated that VRBA method gave significantly higher counts than the rapid method ($P<0.05$). The correlation coefficient of MPN and VRBA data was 0.79, less than that of the raw milk study (4) which was 0.88. However, the intercepts were comparable (0.44 and 0.54). When MPN data were correlated to the RPT data, the correlation coefficient was the lowest obtained (0.59) but the linear relationship was still significant ($P<0.05$). Mean differences between MPN and RPT data were not significant ($P>0.05$). The slightly lower total coliform counts of the RPT found in this study were also observed in the study of frozen raw meats (5). The authors accounted for the differences by the presence of some atypical *E. coli* strains (IMViC-+--), which comprised only 3-5% of the *Escherichia* strains. Even though this study used only typical *E. coli* strains for the inoculum, the presence of atypical *E. coli* was possible in the meat used which originally contained $2.0 \times 10^2$ CFU per g of *E. coli*.

The data from the RPT, despite a higher limit of detection than MPN assay, showed greater precision over a wider range of *E. coli* concentration. Considering the advantages of time-saving, lower material costs and labor involved, the RPT offers a reasonable alternative for MPN and VRBA.
Table 5.1 Comparison of log10 counts of coliforms determined by different methods (P<0.05) on Thai-style fermented pork sausage (n=43).

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<thead>
<tr>
<th>Parameter</th>
<th>VRBA&lt;sup&gt;1&lt;/sup&gt; vs. VRBA&lt;sup&gt;2&lt;/sup&gt;</th>
<th>VRBA&lt;sup&gt;2&lt;/sup&gt; vs. RPT&lt;sup&gt;3&lt;/sup&gt;</th>
<th>RPT vs. MPN&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.94</td>
<td>0.79</td>
<td>0.59</td>
</tr>
<tr>
<td>Slope (b)</td>
<td>0.76</td>
<td>0.91</td>
<td>0.37</td>
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<tr>
<td>Intercept</td>
<td>0.98</td>
<td>0.44</td>
<td>1.84</td>
</tr>
<tr>
<td>t-value&lt;sup&gt;4&lt;/sup&gt; for testing slope = 0</td>
<td>14.33</td>
<td>8.22</td>
<td>3.79</td>
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<tr>
<td>t-statistic&lt;sup&gt;5&lt;/sup&gt; for paired comparison</td>
<td>4.95</td>
<td>2.51</td>
<td>0.34</td>
</tr>
</tbody>
</table>

<sup>1</sup> Plating with Violet Red Bile Agar.
<sup>2</sup> Rapid plating technique.
<sup>3</sup> Most Probable Number Assay.
<sup>4</sup> <sup>t</sup><sub>.05</sub> = 3.67
<sup>5</sup> <sup>t</sup><sub>.05</sub> = 2.05
REFERENCES


In understanding the safety of Thai fermented pork, it is necessary to be aware of the sources of pathogens, the competition among microorganisms during fermentation and in the finished products and the nature of the environment in which the pathogens could proliferate. Recommended actions for prevention of problems must be based on such knowledge. The multiplication and/or survival of *S. aureus*, *E. coli* and trichinae in fermented pork have been evaluated. The fermentation of Nham is a dynamic phenomenon characterized by continuous biochemical, biophysical and microbial changes. The ability of food pathogens to survive, initiate growth, and for *S. aureus* to produce toxins depends on the ability of the organism to overcome the inhibitory environment created during fermentation. Important components of this environment are:

1. initial formulation, including specific salts
2. eventual change in pH, redox potential and $A_w$
3. temperature as related to rates of chemical and physical changes,
4. the initial numbers and types of pathogens,
5. types and numbers of competing microbial flora in the meat; this includes starter cultures.

Under the natural fermentation conditions, if *T. spiralis*
were present in meat that would be made into Nham, fermentation alone would not render the trichinae noninfective. However, addition of starter culture significantly increased the effectiveness of the fermentative process in destroying trichinae infectivity. Prefreezing of the meat prior to the making of Nham will help in accomplishing the safety of the finished product. Freezing at -23°C for 24 hours was not enough to guarantee complete destruction of trichinae larvae. Procedures in the production of Nham that are recommended to safeguard against *T. spiralis* include freezing of raw meat for the specified time if the starter culture is not to be used, the use of at least 1.5% starter culture or, if culturally acceptable, adequate cooking prior to consumption.

*S. aureus* survived well in this type of sausage. However, when starter culture was added, it did not compete successfully. On the other hand, natural fermentation limited the multiplication of *E. coli*. The addition of starter culture enhanced the killing effect and decreased the time it took to eliminate these organisms from the food.

Although other pathogens, such as *Salmonella*, *Campylobacter* and *Yersinia*, are also found often in fresh meat, their prevalence in fermented sausage is yet to be explored. The recommendations derived from the investigation of *S. aureus* and *E. coli* should also prove to be effective for the broader problems.
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APPENDIX A

Trichinae Determination: Artificial Digestion Procedure
APPENDIX A

Trichinae Determination: Artificial Digestion Procedure

20 g sample was placed in a Stomacher bag containing 200 mL of HCl/pepsin solution. The digesting solution was prepared by adding 20 g of pepsin 1:2500 (Sigma, St. Louis, MO) to a 1-L volumetric flask containing 300 mL distilled water. Twelve mL of 1 N HCl were added to the flask and the volume was brought up to 1-L with distilled water. The sample was blended with the pepsin solution in a Stomacher 400 Lab Blender (Tekmar Co., Cincinnati, OH) for 2 min and digested overnight at 37°C in a 600-mL beaker. After incubation, the digested sample was decanted through a No.40 sieve into a 1000-mL graduated cylinder. The beaker was rinsed twice with 100 mL of warm water and the washed solution added to the cylinder and the total volume was made up to 1-L. After 20 min, the top 800 mL were siphoned off. The remaining portion was centrifuged at 500 rpm for 5 min, the supernatant was siphoned off and the centrifugate was resuspended with a known volume of warm physiological saline(40°C) for use as inoculum.
APPENDIX B

Project Data of Distribution of *Trichinella spiralis* in Tissues of Rats Experimentally Infected with the Extractions of Nham (Thai-Style Fermented Pork Sausage) Made from Infected Pork, Frozen and Thawed
Table 1. Distribution of *Trichinella spiralis* larvae in tissues of rats experimentally infected with the extractions of Nham (Thai-style fermented pork sausage) made from infected pork, frozen and thawed.

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APPENDIX C

Project Data and Statistical Analysis of the Enumeration of *Escherichia Coli* Using Violet Red Bile Agar, Most Probable Number and Rapid Plating Technique in Thai-Style Fermented Pork sausages
Table 1. Aerobic plate counts (PCA), lactic acid bacteria (MRS) and Escherichia coli enumeration (VRBA, MPN, RPT) in Thai-style fermented pork sausages.

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### Bivariate Statistics

**Dependent variable is col(1) log10vrba**

**Independent variable is col(2) log10tba (RPT)**

<table>
<thead>
<tr>
<th>Metric</th>
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<tbody>
<tr>
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<td>Number of non-missing rows</td>
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<td>Intercept (a)</td>
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<td>Tail probability for T-test</td>
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**Dependent variable is col(1) log10vrba**

**Independent variable is col(4) log10mpn**

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**Dependent variable is col(4) log10mpn**

**Independent variable is col(2) log10tba (RPT)**

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**Paired T-Test Results**

**Column 1** has the label: log10vrba  
**Column 2** has the label: log10tba(RPT)

<table>
<thead>
<tr>
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<tbody>
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<td>Right tail probability</td>
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<tr>
<td>Two-tailed probability</td>
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</table>

**Paired T-Test Results**

**Column 4** has the label: log10mpn  
**Column 2** has the label: log10tba(RPT)

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**Paired T-Test Results**

**Column 1** has the label: log10vrba  
**Column 4** has the label: log10mpn

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