
**UNIVERSITI PUTRA MALAYSIA**

# *IN-VITRO* INHIBITORY ACTIVITIES *BIFIDOBACTERIUM* SPP. ON *CAMPYLOBACTER JEJUNI* ISOLATED FROM CHICKENS

**ROHAIDAH OMAR**

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**By**

**ROHAIDAH OMAR**

**Thesis Submitted in Fulfilment of the Requirement for the Degree of Master of Science in the Faculty of Veterinary Medicine Universiti Putra Malaysia**

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## *Special Dedication*

*To my husband for his patience, endurance and support throughout this project*
*also to my son Muhammad Ridzwan*

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

# *IN-VITRO* INHIBITORY ACTIVITIES *OF BIFIDOBACTERIUM SPP. ON CAMPYLOBACTER JEJUNI* ISOLATED FROM CHICKENS

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**ROHAIDAH BT. OMAR**

**January 2001**

**Chairman : Associate Professor Saleha bt. Abdul Aziz, DVM, Ph.D**

**Faculty : Veterinary Medicine**

Poultry meat is frequently contaminated with *Campylobacter jejuni* and is considered to be a significant source for campylobacteriosis in man. Various possible strategies to protect chicks against *C. jejuni* colonisation are currently under investigation throughout the world. The use of antagonistic flora has been proposed as one of the approaches to reduce chicken intestinal colonisation by *C. jejuni*. Hence, this study was undertaken to identify *Bifidobacterium* spp. which has been known for its antagonistic activity against many pathogenic bacteria. Bifidobacteria isolated from chickens were screened for the expression of *in-vitro* anti-*C. jejuni* activities, their antibiotic susceptibility and the anti-*C. jejuni* substances involved. Chickens from three broiler farms and four different flocks of village chickens were sampled for four consecutive weeks to determine the presence of *Bifidobacterium* spp. It was found that Trypticase Phytone Yeast (TPY) medium with an adjusted pH of 5.2-5.5 allowed good growth of bifidobacteria and

substantially inhibited growth of other contaminating facultative anaerobic organisms. A total of 57.7% (56/97) of *Bifidobacterium* spp isolates were obtained, of which 23.2% (13/56) were *B. animalis*. The organism was not isolated in one-week-old chicks, however was detectable in two-week-old chicks. There was no significant difference (P>0.05) in variations of the total bifidobacteria isolated with the age of birds or farms in broiler or village chickens. Almost 79% (44/56) of bifidobacteria isolates possessed various degree of *in-vitro* anti-*C. jejuni* activities, and 19.6% (11/56) of the isolates demonstrated marked inhibition. All *B. animalis* isolates (13/13) exerted the inhibitory activities against *C. jejuni*. The antibiotic susceptibility test results indicated that bifidobacteria isolates were susceptible to erythromycin and ampicillin and resistant to nalidixic acid, gentamicin and streptomycin. They showed variable susceptibility to chloramphenicol, tetracycline and neomycin. The inhibitory activities produced by bifidobacteria isolates were possibly ascribed to the production of organic acids in particular acetic acid, and though not clearly shown, perhaps production of bacteriocins-like substances was also partially responsible for the inhibitory effects. However, the possible influence of hydrogen peroxide and other unknown inhibitory substances has not been excluded. Further research is needed to study the existence of such substances and to clarify the protective ability of bifidobacteria isolates, which may have potential to reduce intestinal colonisation by *C. jejuni* in chickens.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

# AKTIVITI PENGHALANG SECARA *IN-VITRO* OLEH *BIFIDOBACTERIUM SPP.* KE ATAS *CAMPYLOBACTER JEJUNI* DARIPADA AYAM

Oleh

**ROHAIDAH BT. OMAR**

**Januari 2001**

**Pengerusi : Profesor Madya Saleha bt. Abdul Aziz, DVM, Ph.D**

**Fakulti : Perubatan Veterinar**

Daging ayam seringkali dicemari oleh *Campylobacter jejuni* dan ia merupakan punca utama kampilobakteriosis dalam manusia. Berbagai strategi sedang dikajisiasat di seluruh dunia untuk melindungi ayam daripada jangkitan *C. jejuni.* Penggunaan flora antagonis telah dicadangkan sebagai salah satu langkah untuk mengurangkan jangkitan *C. jejuni* pada usus ayam. Oleh itu, kajian ini telah dijalankan untuk mengenalpasti *Bifidobacterium* spp. yang memang dikenali dengan aktiviti penghalang terhadap banyak bakteria patogen. Isolat bifidobakteria yang diperolehi daripada ayam, dikaji aktiviti penghalangnya terhadap *C. jejuni* secara *in-vitro*, kepekaan terhadap antibiotik dan unsur-unsur anti-*C. jejuni* yang terlibat. Ayam daripada tiga buah ladang ayam pedaging dan empat kumpulan ayam kampung telah diselidiki selama empat minggu berturut-turut untuk menentukan kehadiran *Bifidobacterium* spp. Kajian mendapati, bahan media Trypticase Phytone Yeast (TPY) dengan pH 5.2-5.5 membolehkan organisma ini

tumbuh dengan baik dan ia mampu menghalang pertumbuhan organisma lain yang bersifat fakultatif anaerob. Daripada kira-kira 57.7% (56/97) isolat *Bifidobacterium* spp. yang diperolehi, 23.2% (13/56) adalah *B. animalis*. Organisma ini tidak ditemui dalam ayam berumur satu minggu tetapi telah ditemui dalam ayam berumur dua minggu. Tiada perbezaan beerti ($P>0.05$) bagi variasi jumlah bifidobakteria yang diasingkan dengan umur atau ladang yang berlainan bagi ayam pedaging atau ayam kampung. Kira-kira 79% (44/56) daripada isolat bifidobakteria tersebut memiliki pelbagai darjah kekuatan aktiviti anti-*C. jejuni* secara *in-vitro*, dengan 19.6% (11/56) daripada isolat mempamerkan aktiviti penghalang ketara. Kesemua isolat *B. animalis* (13/13) menunjukkan aktiviti penghalang terhadap *C. jejuni*. Keputusan ujian kepekaan terhadap antibiotik menunjukkan bifidobakteria yang diasingkan daripada ayam, peka terhadap erythromycin dan ampicillin dan ia rentan kepada asid nalidixic, gentamicin dan streptomycin. Isolat menunjukkan pelbagai kepekaan terhadap chloramphenicol, tetracycline dan neomycin. Aktiviti penghalang yang ditunjukkan oleh isolat bifidobakteria tidak hanya dikaitkan dengan pembentukan asid organik, tetapi kemungkinan juga dikaitkan dengan unsur-unsur "seakan-bakteriosin". Walau bagaimanapun, kebarangkalian pengaruh hydrogen peroksida serta unsur-unsur penghalang lain yang tidak diketahui tidak boleh diketepikan. Penyelidikan lanjut diperlukan untuk mengkaji kewujudan unsur-unsur penghalang tersebut serta mengenalpasti kebolehan memberi perlindungan oleh isolat bifidobakteria ini, yang mungkin berpotensi untuk mengurangkan jangkitan *C. jejuni* pada usus ayam.

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I certify that an Examination Committee met on 9 January 2001, to conduct the final examination of Rohaidah Omar on her Master of Science thesis entitled "*In-vitro* Inhibitory Activities of *Bifidobacterium* spp. on *Campylobacter jejuni* Isolated From Chickens" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

JASNI B. SABRI, DVM, Ph.D,
Associate Professor/Lecturer,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Chairman)

SALEHA ABD. AZIZ, DVM, Ph.D,
Associate Professor/Lecturer,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Member)

MOHD. YAZID ABD. MANAP, Ph.D,
Lecturer,
Faculty of Food Science and Biotechnology,
Universiti Putra Malaysia.
(Member)

ABDUL RAHIM MUTALIB, DVM, Ph.D,
Lecturer,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia.
(Member)

MOHD. GHAZALI MOHAYIDIN, Ph.D,
Professor/Deputy Dean of Graduate School,
Universiti Putra Malaysia

Date: 09 FEB 2001

This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Master of Science.

**MOHD. GHAZALI MOHAYIDIN, Ph.D.**
Professor
Deputy Dean of Graduate School
Universiti Putra Malaysia

Date:

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions

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# LIST OF ABBREVIATIONS

µg: micro gram

µl: micro liter

API Campy: Analytical Profile Index for *Campylobacter*

$CaCl_2$: Calcium chloride 7-hydrate

CE: Competitive Exclusion

FBP Broth: Ferrous Bisulfite Pyruvate broth

$FeCl_3$: Iron (III) chloride 6-hydrate

$H_2SO_4$: Sulfuric acid

HPLC: High Performance Liquid Chromatography

$K_2HPO_4$: Di-pottasium hydrogen orthophosphate anhydrous

M: Molar

MCE: Mucosal Competitive Exclusion

$MgCl_2.6H2O$: Magnesium chloride 6-hydrate

mM: millimolar

N: Normality

NA: Not available

NaCl: Sodium chloride

NaOH: Sodium hydroxide

NT: Not tested

OR: Odd ratio

PYG: Phytone yeast glucose

rpm: rotation per minute

RT: Retention time

Spp.: Species

Subsp.: Subspecies

TPY: Trypticase phytone yeast

$ZnHSO_4.7H_2O$: Zinc sulphate 7-hydrate

# CHAPTER 1

# INTRODUCTION

Campylobacteriosis in human is an important food-borne zoonosis of specific concern to consumers of undercooked poultry, beef and pork. It is a well-recognized food-borne enteritis in human populations with a worldwide distribution. In several developed countries the incidence even exceeds that of salmonellosis (Blaser *et al.*, 1983). In a national survey assessing the prevalence of *Campylobacter jejuni* infection in college campuses in the United States, the organism was isolated 10 times more frequently than *Salmonella* and 46 times more frequently than *Shigella* (Tauxe *et al.*, 1985). In Malaysia, Joseph and co-workers (Joseph *et al.*, 1989) demonstrated that 72.7% of poultry carcasses were positive for *C. jejuni* and *C. coli*. Further work (Saleha and Ibrahim, 1994) showed 81.5% of chicken cloacal swabs were positive for both organisms.

The symptoms and signs of *Campylobacter* enteritis usually include fever, abdominal cramps and diarrhea that last for several days to more than one week. Other clinical manifestations have been described as Guillain-Barre Syndrome (NIAID Workshop Report, 1996). It represents the most common cause of acute neuromuscular paralysis ranging from weakness and tingling sensations in the legs and spread to the arms and upper body, reactive arthritis, meningitis and haemolytic ureamic syndrome (Skirrow, 1992; NIAID Workshop Report, 1996).

Intestinal colonisation of *C. jejuni* in the chicken plays a role in carcass contamination during slaughter. Thus, reducing *C. jejuni* colonisation in chicks can potentially reduce the incidence of *C. jejuni* infections in human. There is considerable interest to control *Campylobacter* infection in commercial poultry flocks, since chicken is the most important vehicle for transmitting the agent to human. Harris and colleagues (Harris *et al.*, 1986) identified poultry consumption as the predominant risk factor for human campylobacteriosis. The report indicated that 48.2% of the *C. jejuni* enteritis cases were associated with handling or consumption of chickens. They also demonstrated that the most frequent serotypes of *C. jejuni* isolated from retail poultry were identical to those found in humans.

Various approaches have been taken towards abating the presence of this human pathogen and it has become a major concern for the poultry industry. However, attempts to protect chicks against *C. jejuni* colonisation have not been consistently successful. Stern *et al.*, (1988) reported that standard preparations of competitive exclusion (CE) and mucosal competitive exclusion (MCE) (Stern, 1993) which were effective against *Salmonella* showed little beneficial effect against colonisation by *C. jejuni*. However, Aho *et al.*, (1992) found benefit in treating chicks with specified antagonistic flora to diminish colonisation by *C. jejuni*. One of the antagonistic flora which received a great deal of attention with respect to the maintenance of a healthy balance of the microflora in the human large intestine is bifidobacteria. Besides lactobacilli and streptococci, the genus *Bifidobacterium* is thought to have numerical advantages on the host's health (Dodd and Gasson, 1994).

*Bifidobacterium* spp. are anaerobic, nonpathogenic, Gram-positive bacteria which have generated increasing interest in the dairy industry. The incorporation of bifidobacteria into human diet corresponds to the emergence of a new generation of fermented dairy products, which exploit the beneficial effect of bacteria of human origin on intestine metabolism (Roy *et al.*, 1994). Bifidobacteria are found to be a predominant component of the intestinal flora in breast-fed infants (Ishibashi and Shimamura, 1993). These bacteria are also present in the intestine of various animals (Mitsuoka, 1984). The organism is thought to exert various beneficial effects, which have been clearly demonstrated both in animals and humans. The majority of the proposed physiological effects of bifidobacteria pertain to the improvement of intestinal flora by preventing colonisations by pathogens both *in-vivo* and *in-vitro*, including *Salmonella, Shigella, Clostridium, Bacillus cereus, Staphylococcus aureus, Candida albicans* and *C. jejuni* (Anand *et al.*, 1985; Tojo *et al.*, 1987). Other effects include amelioration of diarrhoea and constipation, immunity activation, vitamin production and antitumor activity (Sekine *et al.*, 1995).

The use of bifidobacteria in pharmaceutical preparations has become widely accepted. The bifidobacteria has been used as an adjunctive treatment for gastrointestinal infections (Tojo *et al.*, 1987). According to the study, *B. breve* were effective in eradicating *C. jejuni* and restoring normal intestinal flora of diarrheic patients. In other studies, oral application of bifidobacteria preparation seemed to reinforce recovery of normal intestinal flora and alleviate clinical symptoms in weaned puppies, early weaned bull calves and suckling pigs (Kimura

*et al.*, 1980). It has been ascribed that the undissociated acid which is present in increasingly higher proportions as pH decreases, is the antibacterial agent. Unlike other bacterial group such as lactobacilli (Ibrahim and Bezkorovainy, 1993) limited study has been done on the nature of the antibacterial substances produced by bifidobacteria of animal origin particularly those isolated from chickens.

Considering these reasons, the objectives of the present study are:

a) to determine the presence of *Bifidobacterium* spp. in broiler and village chickens.

b) to screen for the expression of *in-vitro* inhibitory activities of *Bifidobacterium* spp. against *Campylobacter jejuni* of broiler chickens.

c) to determine the antibiotic susceptibility of bifidobacteria isolates.

d) to study the antibacterial properties of bifidobacteria isolates.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Public Health Significance of *Campylobacter jejuni*

*Campylobacter jejuni* is the most significant of the three thermophilic *Campylobacter* species, and is responsible for intestinal colonisation in poultry and food-borne enteritis in human. Awareness of the public health implications of *Campylobacter* infections has evolved over more than a century (Shane, 1992). It has become the leading cause of gastroenteritis in the developed world and is often acquired by ingestion of infected poultry products (Shanker *et al*., 1982; Blaser *et al*., 1983). The significance of campylobacteriosis as a food-borne pathogen is confirmed by several prevalence studies. Based on a USA data, it is estimated that 60,000 to 170,000 clinical cases occurred in that country during 1982. It is further confirmed that 2000 fatalities resulted from campylobacteriosis, mainly confined to neonates and infants up to 3 months of age, geriatric and immuno-suppressed patients (Blaser *et al*., 1983). The Center for Disease Control and Prevention (CDC) of the USA estimated that there are 2.5 million cases of campylobacteriosis with over 200 deaths annually in the United States (Stern and Robach, 1995). The annual cost of campylobacteriosis in the United States as applied to direct and indirect cost for medical expenses, and the loss attributed to mortality is estimated to range from $700 million to $1400 million (Shane, 1992). In the UK, the cost per case of *Campylobacter* infection has been estimated to be £275 (about US$480) from health care and loss of

productivity is likely to be similar in the USA (Skirrow and Blaser, 1992). In countries such as Malaysia and Singapore, the few published reports give a low isolation rate for *Campylobacter* but the true incidence may be 5-10 times greater than that of industrialised countries (Puthucheary *et al.*, 1994).

## 2.2 Occurrence of *Campylobacter jejuni* in Poultry

Various *Campylobacter* species are recognised in relation to veterinary public health. Of these, *C. jejuni* and *C. coli* are the most frequently isolated from human cases of gastroenteritis (Report, 1993). The observations of Butzler and Oosterom (1991) relating to similarity of *Campylobacter* spp. isolates derived from human patients and poultry had revealed a significant association between the consumption of poultry meat and *Campylobacter* enteritis.

The gastrointestinal tract of chickens and hens, are generally colonised by *Campylobacter* spp. (Lior, 1984) but *C. jejuni* should not be regarded as part of the normal intestinal microflora of poultry (Van de Giessen *et al.*, 1993). In poultry, the incidence of *Campylobacter* infection increases with age (Lindblom *et al.*, 1986). Generally, *C. jejuni* is apathogenic in poultry, although newly hatched chicks and turkeys may develop a transient diarrhoea following infection (Shane, 1992). According to Stern and Meinersmann (1989), infected chickens are usually healthy carriers of *C. jejuni* and they are typically asymptomatic.