SCREENING OF PROBIOTIC ORGANISMS FOR THEIR ANTAGONISTIC ACTIVITY AND NUTRITIONAL POTENTIALITY

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In partial fulfillment of the requirements for the degree of Master of Philosophy in Botany

Submitted by

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Certificate

This is to certify that the research work embodying the results reported in this thesis entitled "Screening of Probiotic organisms for their antagonistic activity and nutritional potentiality" by Sanjida Binte Zuha, has been carried out under our supervision in the laboratory of microbiology, Department of Botany, University of Dhaka and in the Institute of Nutrition and Food Science, University of Dhaka. It is further certified that the research work presented here is original and suitable for submission for the partial fulfillment of the degree of Master of Philosophy in Botany. It can be mentioned here that this work has not been submitted anywhere else for any other degree.

13/03/2012

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DEDICATED TO

MY PARENTS, TEACHER M.R. KHAN, LAB MATES

& MICROBES WHO HELP US TO LIVE

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-Author

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List of Abbreviations:

α	*Alfa*
β	*Beta*
%	*Percentage*
&	*And*
^0C	*degree celsius*
Co.	*Company*
Conc.	*Concentration*
d	*day*
e.g	*Exempli gratia (for example)*
ed	*Edition*
Ed	*Editor*
et al.	*et alibi (with others)*
etc.	*Etcetra*
Fig.	*Figure*
γ	*Gama*
g	*gram*
h	*Hour*

List of Abbreviations:

hrs	*Hours*
i.e.	*Id est (that is)*
Inc.	*Incorporation*
LAB	*Lactic acid bacteria*
L or l	*Litre*
Ltd.	*Limited*
MH	*Müler Hinton*
MRS	*De man Rogosa Sharpe*
M.R	*Methyl-Red*
mg	*Miligram*
ml	*mililitre*
N_2	*Nitrogen*
NaCl	*Sodium Chloride*
nm	*Nano meter*
No.	*Number*
O.D.	*Optical density*
PDA	*Potato dextrose Agar*
pH	*Negative logarithm of hydrogen ion concentration.*

List of Abbreviations:

pp	*Pages*
Sp	*Species (Singular)*
UV	*Ultra Violet*
V.P.	*Voges-Proskauer*
Viz.	*Videli (namely)*
W/V	*Weight per Volume*
YMA	*Yeast extract-Malt extract agar*
μ	*Micron*
μg	*Microgram*
μm	*Micrometer*
ρ	*para*

Abstract

Abstract

In an attempt to study the components and properties of probiotic organisms in Bangladesh, samples were collected from different parts of the country and these were used as sources. The collected samples included fermented dairy products (yogurt, cheese etc.), fermented fruits and vegetables. The screening procedures yielded organisms with distinct characteristics and thus could be easily classified as probiotic.

In all 200 organisms were isolated and after repeated screening 43 were finally selected for detail study. The history, morphological, physiological, biochemical and cultural characters of the selected organisms were considered and compared with the standard description in the available references for their provisional identification.

The work was performed in four steps, a). Isolation of probiotic microorganisms, b). Study their characteristics, c). Testing for their antimicrobial activities and finally d). Testing for their effect during fermentation of selected fruits and vegetables.

For the purpose of isolation, selective media viz. Modified MRS medium, Bifidobacterial medium, Leuconostoc medium and Lee's agar medium were used. As the probiotic organisms are generally considered as anaerobic or microaerophilic, the isolation was carried out both under anaerobic and aerobic conditions.

34 isolates resembled the genus Lactobacillu, while other isolated genera were Leuconostoc, Streptococcus and Pediococcus. Among the 43 organism, 34

belonged to Lactobacillus genus such as. L. casei subsp. pseudoplantarum, Lactobacillus coryniformis subsp. torquens, Lactobacillus amaylophilus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus jensenii, Lactobacillus farcimini, Lactobacillus crispatus, Lactobacillus plantarum, Lactobacillus kefir, Lactobacillus sharpeae, Lactobacillus bifermentans, Lactobacillus bavaricus, Lactobacillus hilgardii, Lactobacillus casei subsp. casei, Lactobacillus salivarius, Lactobacillus maltaromicus, Lactobacillus casei subsp. rhamnosus, Lactobacillus collinoides and Lactobacillus yamanashiensis. 4 organisms were closely related to Leuconostoc mesenteroides subsp. mesenteroides, Leuconostoc mesenteroides subsp. cremoris, Leuconostoc paramesenteroides and Leuconostoc lactis. 2 organisms showed similarity with the genus Streptococcus and these were Streptococcus lactis and Streptococcus parvulus. One organism was identified as Pediococcus urinaeequi.

The remarkable fermentation capability of the isolated probiotic organisms was indicative of being helpful in our digestion process while their antimicrobial activity clearly showed their possible role in our GI tract to relieve us from undesirable microbes.

Introduction

Probiotics:

Probiotic is derived from Greek words *προ* and *βιοτοσ* and translated as ''for life'' (Hamilton-Miller *et al.* 2003). The word ''probiotic'' was initially used as an antonym of the word ''antibiotic''. The origin of the first use can be traced back to Kollath (1953), who used it to describe the restoration of the health of malnourished patients by different organic and inorganic supplements. A year later, Vergin (1954), first introduced the term "Probiotic" as they are defined nowadays, when he compared in his manuscript "Anti- und Probiotika" the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population, with factors ("Probiotika") favorable to the gut microflora. Similarly, Kolb (1955) recognized detrimental effects of antibiotic therapy and proposed the prevention by probiotics.

Evolution of probiotic concept:

The origin of probiotic foods dates back to the dawn of civilization; they are mentioned in the Bible and the sacred books of Hinduism. In a Persian version of Old Testament (Genesis 18:8) it states that "Abraham owed his longevity to the consumption of sour milk." The concept of probiotic foods was introduced long ago with Hippocrates and his motto ''Let food be your medicine''. Recently the body of evidence started to support the hypothesis that diet may play an important role in modulation of important physiological functions in the body.

The work of numerous scientists, mainly microbiologists, resulted in important developments and expansion of knowledge pertaining to the microbiology of the human body. Escherich (1885) was the first to recognize the importance of examining bacteria

appearing in normal feces and the intestinal tract, and consequently understanding the physiology of digestion and the pathology and therapy of intestinal diseases of microbial origin. In 1900, two microbiologists, Tissier and Moro, reported their findings of isolates from the feces of breast-fed infants. Tissier noted that the anaerobically cultured organism had, in general, staining reactions and morphological appearance similar to those of lactobacilli; however, many of them appeared in bifurcated forms. Thus, he named them *Bacillus bifidus*. Similarly, Moro (1900) postulated that the isolate, which he termed *Bacillus acidophilus* due to its unusual acid tolerance, was derived from the mother's breast and normally resided in the neonate's oral cavity and intestinal content. Later, Tissier (1908) also showed that *B. bifidus* was the predominant organism in the faeces of breast-fed infants approximately three days postpartum as opposed to bottle-fed neonates, which predominantly contained *B. acidophilus*.

Louis Pasteur's revolutionary notion that microbes are important causative agents in human disease brought about a celebrated paradigm shift in prevention and treatment of infectious disorders in the form of antiseptic practices, vaccines and later antimicrobial drugs. Pasteur's junior colleague and coworker Elie Metchnikoff later proposed that, in addition to causing disease, certain lactic acid-producing bacteria found in fermented milk products might have health-promoting effects. He was the first to recommend ingestion of living beneficial microorganisms. Metchnikoff's theory thus initiates the concept of 'probiotic'. Nobel Laureate Ilya Metchnikoff noticed that Bulgarian peasants had an average life-span of 87 years, exceptional for the early 1900s, and that four out of every thousand lived past 100 years of age. One of the major differences in their lifestyle in comparison with the contemporary diet was a large consumption of fermented milk. In

his well known auto intoxication theory (Metchnikoff 2004), Metchnikoff suggested that a human body was slowly poisoned by toxins present in the body produced by pathogens in the intestine and body's resistance steadily weakened by proliferation of enteric pathogens, all of which were successfully prevented by the consumption of sour milk and lactic acid producing bacteria. His work was based on an organism previously isolated by Grigoroff (1905), who cultivated it from ''podkvassa'' used as a starter for production of the Bulgarian ''kiselo mleko'' (''sour milk'' or ''yahourth'') and called it *Lactobacillus bulgaricus*. In the process, Grigoroff also identified another organism, *Streptococcus thermophilus*, which received no attention since it was considered a pathogen at that time. Metchnikoff's experiments led him to believe that *L. bulgaricus* could successfully establish itself in the intestinal tract and prevent multiplication and even decrease the number of putrefactive bacteria

Scientists continued to investigate possible benefits of bacteria to the human health. Consequently, certain strains of Lactobacillus acidophilus were isolated and found to be capable of colonizing human digestive tract where they exerted appreciable physiological activity. Rettger and Cheplin (1920b) reported that feeding of milk or lactose to rats or humans led to a transformation of the intestinal microflora resulting in predominance of acidophilus and bifidus type culture. These findings stimulated commercial interest in products fermented by *L. acidophilus*. Other researches followed suit with Minoru Shirota in Japan, who recognized the importance of the preventive medicine and modulation of the gastrointestinal microflora. In 1930, he isolated a Lactobacillus strain capable of surviving the passage through the gastrointestinal tract. The culture identified *as Lactobacillus casei* strain *Shirota* was successfully used for the production of the

fermented dairy product called ''Yakult'', which initiated the foundation of the same company in 1935 (Yakult 1998). In the period between late 1930s and late 1950s, the research in this area lost its pace likely due to war and depression, the world was facing at that time. The rejuvenated interest in the intestinal human microflora was seen in the late 1950s and early 60s that led to the introduction of the probiotic concept.

Definitions of Probiotics:

Probiotics has been redefined throughout the years as more scientific knowledge and better understanding on its relationship between intestinal health and general well being has been gained. The following are definitions of probiotics derived through times.

Lilly and Stillwell in 1965 defined probiotics as "Growth promotiong factors produced by microorganism."

Fujii and Cook (1973) described probiotics as "Compounds that either stimulated microbial growth or improved the immune response of the host without inhibiting the growth of the culture in vitro."

Parker (1974) suggested "An interaction between microorganisms with the host.

Organisms and substances with beneficial effects for animals by influencing the intestinal microflora."

Fuller in 1989 defined it as "A live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance." However his definition was more applicable to animals than to humans.

Havenaar and Huis Int Veld (1992) said probiotics are "A mono- or mixed culture of live microorganism which, applied to animal or man, affect beneficially the host by improving the properties of the indigenous microflora."

According to Salminen (1996) "Live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host." In 1996 Schaafsma defined probiotic as "Living microorganisms which, upon ingestion in certain numbers, exert health."

ILSI (International Life Sciences Institute) Europe Working Group (1998) (Salminen *et al.* 1998):"A viable microbial food supplement which beneficially influences the health of the host."

Diplock *et al.* (1999) puts it as "Probiotic food is functional if they have been satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being and/ or reduction in the risk of diseases.'

Naidu *et al.* (1999) said "A microbial dietary adjuvant that beneficially affects the host physiology by modulation mucosal and systematic immunity as well as the host physiology by modulation mucosal and systematic immunity, as well as improving nutrition and microbial balance in the intestinal tract."

Tannock in 2000 observed that consumption of probiotics was not associated with any drastic change in the intestinal microbiota composition, and thus proposed an alternative definition; "Microbial cells which transit the GI tract and which in doing so, benefit the health of consumer."

Schrezenmeir and de Vrese in 2001 defined probiotics as "A preparation of product containing viable, define microorganism in sufficient number, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in the host."

FAO/WHO (Food and Agriculture Organization and World Health Organization) (2001) and Reid *et al.* (2003) concentrated exclusively on its health purpose: "Live microorganism when administered in adequate amounts confer a health benefit on the host."

Selection criteria of probiotic microorganisms:

Gordon *et al.* (1957) noted that for achieving successful outcome of the lactobacilli therapy was necessary for the preparation to fulfill following requirements: the culture must be a normal inhabitant of the intestine, non-pathogenic, and must be capable of efficient gut colonization and delivered in substantially high concentrations (10^7–10^9 cfumL^{-1} of a product). More recently, an FAO/WHO (2001) expert panel suggested that the specificity of probiotic action is more important than the source of microorganism. Although numerous criteria have been recognized and suggested (Mattila-Sandholm *et al.* 2002; Ouwehand *et al.* 1999), a general agreement exists with regard to key selection criteria listed in Table 1 (FAO/WHO 2002).

Table 1: Key and desirable criteria for the selection of probiotics in commercial applications (adapted from Shah 2006; Morelli 2007)

General	Property
Safety criteria	Origin Pathogenicity and infectivity Virulence factors—toxicity, metabolic activity and intrinsic properties, i.e., antibiotic resistance
Technological criteria	Genetically stable strains Desired viability during processing and storage Good sensory properties Phage resistance Large-scale production
Functional criteria	Tolerance to gastric acid and juices Bile tolerance Adhesion to mucosal surface Validated and documented health effects
Desirable physiological criteria	Immunomodulation Antagonistic activity towards gastrointestinal pathogens, i.e., *Helicobacter pylori, Candida albicans* Cholesterol metabolism Lactose metabolism Antimutagenic and anticarcinogenic properties

Probiotic microorganisms:

Although various strains of lactic acid bacteria have been described as probiotic microorganisms, but many are too sensitive to intense acidity and presence of bile salts in the animal's GI tract, so they die en route to the gut (Hekmat and Reid 2006). The majority of probiotic products available in the marketplace contain species of *Lactobacillus* and *Bifidobacterium* (FAO/WHO 2001). According to Holzpfel and Schillinger (2002), other lactic acid bacteria with probiotic properties belong to genera: *Enterococcus* and *Sporolactobacillus*. Nonlactic acid microbes- *Propionibacterium* and *Saccharomyces* are also considered as probiotic microorganisms.

Probiotic potential also found in the genera- *Lactococcus*, *Leuconostoc*, *Streptococcus* and *Pediococcus* (Pestka *et al.* 2001).

Table 2: Microorganisms used as Probiotic (Knorr 1998; Alvarez-Olmos and Oberhelman 2001; Senok *et al.* 2005; Santosa *et al.* 2006 and Senok 2009).

Lactic acid bacteria			Non-lactic acid
Lactobacillus	*Bifidobacterium*	Other micorganisms	
L. acidophilus	*B. bifidum*	*Streptococcus thermophilus*	*Propionibacterium freudenreichii*
L. casei	*B. longum*	*Enterococcus faecalis*	
L. johnsonii	*B. brevis*	*E. faecium*	*Saccharomyces cerevisiae*
L. rhamnosus	*B. infantis*	*Sporolactobacillus inulinus*	
L. thermophilus	*B. animalis*	*Lactococcus lactis* ssp. *cremoris*	*Saccharomyces boulardii*
L. reuteri	*B. adolescentis*	*Lactococcus lactis* ssp. *lactis*	
L. delbrueckii subsp. Bulgaricus	*B. lactis*	*Leuconostoc mesenteroides* ssp. *dextranicum*	
L. crispatus		*Pediococcus acidilactici*	
L. fermentum			
L. gasseri			
L. lactis			
L. plantarum			

Source of Probiotic microorganisms:

Lactic acid bacteria (LAB) have been used in the preservation of milk and vegetables for centuries (Sgouras *et al.* 2004). Today, a wide variety of strains including LAB, Propionic acid bacteria (PAB) and Bifidobacteria are industrially used as starter cultures,

cocultures or bioprotective cultures to improve preservation, flavors and texture of milk, vegetable meat and cereal products. The scientific basis of improved preservations based on fermentation in which

a) The pH value is lowered.

b) The amount of available carbohydrates is reduced and

c) Many antimicrobial compounds are produced by fermentation bacteria.

Metchnicoff proposed that the acid-producing organisms in fermented dairy products could prevent "fouling" in the large intestine and thus lead to a prolongation of the life span of the consumers (Metchnikoff 1908). As the interest on probiotic gradually increased, it becomes eminent to diversify the sources in the search for more effective, novel probiotic microorganisms. For this purpose different samples e.g. Dairy and nondairy fermented products, animal intestine, milk, infant's feces etc.

I. Microorganisms of animal origin:

Microorganisms isolated from human milk and breast-fed infants are especially attractive as probiotics as they fulfill some of criteria generally recommended, such as human origin and a history of safe prolonged intake by infants. Among the bacteria isolated from human milk, species such as *Bifidobacerium longum, Bifidobacterium breve* (Martin et al. 2009), *Lactobacillus gasseri, L rhamnosus, L salivarius, L reuteri, L plantarum, L fermentum* and *Enterococcus faecium* (Martin et al. 2007). Beneficial microorganism could also be found among the colonizing populations from mouth, esophagus and stomach (Bik *et al.* 2006).

II. Dairy Fermented Food:

The fermentation of dairy foods presents one of the oldest methods of long-term food preservation. The origin of fermented milk can be traced back long before the Phoenician era and placed in the Middle East. Traditional Egyptian fermented milk products, Laban Rayeb and Laban Khad, were consumed as early as 7000 BC (Kosikowski and Mistry 1997). The tradition of fermenting milk was spread throughout the East Europe and Russia by the Tartars, Huns and Mongols during their conquests. With the increased data about benefits of probiotics for human health and treatment, dairy fermented foods drew extra attention. Name of some familiar dairy foods with their dominating microbial community are enlisted below:

Acidophilus milk (Heller 2001) - acidophilus milk is produced by fermentation with *Lactobacillus acidophilus.* Before fermentation, milk go through the intensive heat treatment to yield sterile milk which is necessary for successful fermentation because *L. acidophilus* acidifies slowly and thus can be readily competed out by contaminating bacteria.

Bulgerian buttermilk- *L delbrueckii* subsp. *Bulgaricus*

Kefir (Heller 2001)-An important ideal probiotic dairy product may be kefir because probiotic strains have been isolated from several members of typical microflora e. g.

Lactobacilli- *L. kefir, L. acidophilus, L.casei, L. reuteri, L. fermentum, L. brevis, L. buchneri.*

Lactococci- *Lactococcus lactis, Leuconostoc lactis, Leuconostoc mesenteroides.*

Yeast- *Candida kefir, Saccharomyces cerevisiae, Kluyveromyces* etc.

Acetic acid bacteria- *Acetobacter pasteurianus.*

Kumiss (James 2000) - *Lactobacillus leichmannii, "Torula"* spp, *L. delbrueckii* subsp. *bulgaricus*.

Taette (James 2000) - *Streptococcus lactis* var. *taette*

Tarhana (Mahasneh and Abbas 2010) - Turkish traditional fermented food made up of dried mixture of cracked wheat yogurt or fermented milk. Tarhana is a rich source of probiotic lactic acid bacteria.

Kashik (Mahasneh and Abbas 2010) - is like tarhana, it is Jordanian traditional fermented food made up of boiled wheat, thick curd of goat or sheep yoghurt or fermented milk, solar drying where slow fermentation subjected to take place. Kashik is also the source of probiotic bacteria (Tamime and O'connor 1995).

Yogurt (Heller 2001 and Doleyres and Lacroix 2005)- Yogurt like products are manufactured with different textures. Natural-set yogurt, stirred yogurt and drinking yogurt differ in their content of nonfat solids (16-18%, 13-14% and 11-12%, respectively). Classic yogurt is produced with a thermophilic protosymbiotic culture of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, the so-called mild yogurt (pH-4.3-4.0) is produced with a themophilic culture of *S. thermophilus* and a lactobacillus species, usually *L. acidophilus*. *Bifidobacterium animalis* subsp. *lactis* is the most commonly used Bifidobacterium species in yogurt production. Mild yogurt improves survival of bifidobacteria compared to traditional yogurt (pH-3.7-4.3). probiotic lactobacilli and bifidobacteria are not highly proteolytic and do not grow well in milk alone.

Cheese (Heller 2001 and Lee and Salminen 2009) - Coagulation in cheese is achieved through the proteolytic action of rennet. Less rennet is added for fresh cheese (e.g.

cottage cheese) than for ripened or matured cheeses (e.g. cheddar cheese). Mesophilic microorganisms inoculated in fresh cheeses and in case of mature cheeses, mesophilic or thermophilic bacteria are used as starter culture.

Matured cheeses - Lactobacilli: *L. casei, L. paracasei, L. rhamnosus, L. helveticus*

Lactococci: *Streptococcus thermophilus*

Bifidobacteria: *B. animalis* subsp. *lactis*, *B. adolescentis, B. breve, B. longum*

Fresh cheeses- Lactobacilli: *L. rhamnosus, L. casei*

Lactococci: *Lactococcus* spp, *Leuconostoc* spp.

Bifidobacteria: *B. infantis, B. bifidum, B. animalis* subsp. *lactis*.

Different types of non fermented dairy food matrix also considered as reservoir of probiotics in commercial level e.g. Ice cream, Dairy desserts, Dry products such as infants formula and nutritional powders, sweet acidophilus milk, sweet AB milk etc.

Ice-cream: *L. johnsonii, L. casei, L. rhamnosus, B. animalis* subsp. *lactis*, *B. longum, B.bifidum*.

Sweet acidophilus milk: *L. acidophilus*.

Sweet AB milk: *L. acidophilus* and *Bifidobacterium* spp.

Dairy desserts: L. paracasei, L acidophilus.

Infant's formula and nutritional powders: *L. rhamnosus, L. acidophilus* and *B. animalis* subsp. *lactis*.

III. Non dairy Fermented Food:

From beginning of probiotics, most researches are conducted on the microorganisms which are mostly of human or animal origin. However, some studies show that strains

recognized as probiotics are also found in non dairy fermented foods (Schrezenmeir and De Vrese 2001). Moreover, over centuries fermentation has been used to preserve, improve the quality or modify the flavor of cereals, fruits, vegetables, legumes and meat. As fermentation process involves mixed cultures such as yeast, lactic acid bacteria (LAB) and fungi (Blandino *et al.* 2003), traditional fermented foods are a plentiful source of microorganism and some of them show probiotic characteristics (Table 3) (De Valdez *et al.* 1990; Ashenafi and Busse 1991; Harris *et al.* 1992; Botes *et al.* 2007, Lei and Jacobsen, 2004, Psani and Kotzekidou 2006, Todorov et al. 2008).

Commercially important probiotics:

Probiotic cultures have been exploited extensively by the dairy industry as a tool for the development of novel functional products. Traditionally, probiotics have been incorporated in to yoghurt; however, a number of carriers for probiotics have been examined recently including edible spreads and meat (Arihara *et al.* 1998) in addition to other products of dairy origin, i.e., cheese (Ong *et al.* 2006) or cheese-based dips (Tharmaraj and Shah 2004). Probiotic organisms are also available commercially in milk, sour milk, fruit juices, ice cream, single shots and oat-based products. Lunebest, Olifus, Bogarde, Progurt are only some examples of commercial fermented dairy products with probiotics available on the international market with a steady increase in the market shares. The consumption of functional dairy products across West Europe, United States and Japan rose by 12% since 2005 (Zenith International 2007). Probiotic products are very popular in Japan as reflected in more than 53 different types of probiotic-containing

products on the market. Commercial cultures used in these applications are mentioned in Table 4.

Applications of probiotics in human health:

Probiotics are defined as possessing valuable factors contributing to the health and well-being of hosts, including humans. It is thought that consumption of a sufficient amount (more than 10^9 CFU) per day of probiotics will be sufficient to balance the human GI micro-ecosystem by replacing harmful pathogens and reinforcing the natural defence mechanisms (Ouwehand *et al.* 2002). However, probiotics are not a uniform gtroup of microorganisms with health benefits; hence, the efficacy and safety of each specific species and strain should be assessed individually rather than as a group of probiotics. As probiotics by definition impart health benefits to the host, it is important to review the scientific documentation and basis for such benefits. Based on research reports, it has been demonstrated that all probiotic health effects are strain, dose, disease and possibly host dependent (Lee and Salminen 2009). Some of the established and potential health effects are listed in table 5.

Table 3. Non dairy probiotic traditional fermented foods.

Product	Probiotic microorganisms	Substrates
Adai	LAB	Cereal, legume
Agbelima	*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, *Leuc. Mesenteroides*	Cassava
Atole	LAB	Maise
Ben-saalga	LAB	Pearl millet
Boza	*Lb. plantarum*, *Lb. brevis*, *Lb. rhamnosus*, *Lb. fermentum*, *Leuc. mesenteroides* subsp. *Dextranium*	Cereals
Dosa	*Leuc. mesenteroides*, *Lb. fermentum*, *Sacch. Cerevisiae*	Rice and Bengal gram
Idli	*Leuc. mesenteroides*, LAB, yeast	Cereal, legume
Ilambazi lokubilisa	LAB	Maize
Kecap	LAB	Wheat, soybeans
Kenkey	*Lb. casei*, *Lb. lactis*, *Lb. plantarum*, *Lb. brevis*, *Lb. acidophilus*, *Lb. fermentum*, *Lb. casei*, yeast	Maize
Kimchi	*Lb. plantarum*, *Lb. curvatus*, *Lb. brevis*, *Lb. sake*, *Leuc. Mesenteroides*	Vegetables
Kishk	LAB	Cereal and milk
Kisra	*Lactobacillus* sp., *Lb. brevis*	Sorghum
Koko	*Lb. fermentum*, *Lb. salivarius*	Millet
Mahewu	*Lb. bulgaricus*, *Lb. brevis*	Maize
Mawe	*Lb. fermentum*, *Lb. brevis*, *Lb. salivarius*, *Sacch. Cerevisiae*	Maize
Ngari	*Lactococcus lactis* subsp. *cremoris*, *Lactococcus plantarum*, *Enterococcus faecium*, *Lb. fructosus*, *Lb. amylophilus*, *Lb. coryniformis* subsp. *torquens*, and *Lb. plantarum*	Fish
Nan	LAB *Saccharomyces cerevisiae*	White wheat flour
Ogi	*Lb. plantarum*, *Lb. fermentum*, *Leuc. mesenteroides*, and *Sacch. Cerevisiae*	Maize
Sauerkraut	*Leuc. mesenteroides*, *Lactococcus lactis*, LAB	Cabbage
Som-fug	LAB	Fish
Tarhana	*Streptococcus thermophilus*, *Lb. bulgaricus*, *Lb. plantarum*	Parboiled wheat meal and yogurt
Tempeh	LAB, *Lb. plantarum*	Soybean
Uji	LAB	Maize, sorghum cassava, finger millet

Table 4: Some probiotic strains used in commercial applications (adapted from Holm, 2003)

Strain	Source
L. acidophilus LA1/LA5 *L. delbrueckii* ssp. *bulgaricus* Lb12 *L. paracasei* CRL431 *B. animalis* ssp. *lactis* Bb12	Chr. Hansen
L. acidophilus NCFM® *L. acidophilus* La *L. paracasei* Lpc *B. lactis* HOWARU™/Bl	Danisco
L. acidophilus LAFTI®L10 *B. lactis* LAFTI®B94 *L. paracasei* LAFTI® L26	DSM Food Specialties
L. johnsonii La1	Nestle
L. acidophilus SBT-20621 *B. longum* SBT-29281	Snow Brand Milk Products Co. Ltd.
L. rhamnosus R0011 *L. acidophilus* R0052	Institute Rosell
L. casei Shirota *B. breve* strain Yakult	Yakult
B. lactis HN019 (DR10) *L. rhamnosus* HN001 (DR20)	Foneterra
L. plantarum 299V *L. rhamnosus* 271	Probi AB
L. casei Immunitas *B. animalis* DN173010 (Bioactiva)	Danone
L. rhamnosus LB21 *Lactococcus lactis* L1A	Essum AB
L. reuteri SD2112	Biogaia
L. rhamnosus GG1	Valio Dairy
L. salivarius UCC118	University College Cork
B. longum BB536	Morinaga Milk Industry Co. Ltd.
L. acidophilus LB	Lacteol Laboratory
L. paracasei F19	Medipharm

Table5: Some of the established and potential health benefits of probiotic microorganisms (adapted from Shah 2006).

Health effect	Mechanism
Scientifically established **Alleviation of lactose intolerance**	Delivery of intracellular b-galactosidase into human gastrointestinal tract
Prevention and reduction of symptoms of rotavirus and antibiotic associated diarrhea;	Competitive exclusion Translocation/barrier effect Improved immune response
Potential **Treatment and prevention of allergy (atopic eczema, food allergy)**	Translocation/barrier effect Immune exclusion, elimination and Regulation
Reduction of risk associated with mutagenicity and carcinogenicity	Metabolism of mutagens Alteration of intestinal micro-ecology Alteration of intestinal metabolic activity Normalization of intestinal permeability Enhanced intestinal immunity
Hypocholesterolemic effect	Deconjugation of bile salts
Inhibition of Helicobacter pylori and intestinal pathogens	Competitive exclusion Barrier effect Production of antimicrobial compounds
Prevention of inflammatory bowel Diseases	Competitive exclusion Improvement of epithelial tight junctions Modification of intestinal permeability Modulation of immune response Production of antimicrobial products Decomposition of pathogenic antigens
Stimulation of immune system	Recognition by toll-like receptors—induction of innate and adaptive immunity: _ downregulation of pro-inflammatory cytokines and chemokines _ upregulation of phagocytic activity _ regulation of Th1/Th2 balance

Lactose Maldigestion: The decline of the intestinal β-galactosidase (β-gal or commonly known as lactase) activity is a biological characteristic of the maturing intestine in the majority of the world's population (de Vrese *et al.* 2001). Lactose upon ingestion is hydrolyzed by lactase in the brush border membrane of the mucosa of the small intestine into constitutive monosaccharides, glucose and galactose, which are readily absorbed in the blood stream. However, the activity of intestinal lactase in lactose intolerant individuals is usually less than 10% of childhood levels (Buller and Grand 1990). This decline, termed hypolactasia, causes insufficient lactose digestion in the small intestine, characterized by an increase in blood glucose concentration or hydrogen concentration in breath upon ingestion of 50 g lactose, conditions designated as lactose maldigestion (Scrimshaw and Murray 1988). Hypolactasia and lactose malabsorption accompanied with clinical symptoms, such as bloating, flatulence, nausea, abdominal pain and diarrhoea, are termed lactose intolerance. Symptoms are caused by undigested lactose in the large intestine, where lactose is fermented by intestinal microflora and osmotically increases the water flow into the lumen. The severity of the symptoms depends primarily on the size of the lactose load ingested.

Natural and denatured yogurt improved lactose digestion in lactose maldigesters. The effect of natural yogurt is higher than that of heat treated yogurt. Natural yogurt almost abolishes the symptom of lactose maldigestion in lactose maldigesting person, for lactose intake of up to 20g. β-galactosidase may not be the sole and major factor involved in the digeston of lactose in milk products. Other factors, such as intracellular β-galactosidase released in the small intestine and the yogurt matrix may play an important role in improving lactose digestion (Lee and Salminen 2009).

Prevention and treatment of oral infection and dental caries: Acidogenic oral bacteria such as Streptococci and Lactobacilli are associated with the presence and onset of dental caries (Varcoe *et al.* 2003). The caries bacteria colonize acidic environments, such as fissures and interdental space and produce acid from fermentable sugars. Overgrowth of oral yeast is a common problem among the elders.

There was a suggestion that temporary colonization of a nonlactose and slow sugar fermenting bacteria, which are able to inhibit the caries pathogens, may prevent caries in children (Wagner *et al.* 2000). Temporary colonization of probiotic bacteria in the oral cavity may competitively exclude the oral yeast (Asahara *et al.* 2001).

L. rhamnosus GG is used to prevent dental caries (Dose-5-10×10^9 cfu/mL milk) and oral candida (Dose-50g cheese) (Wagner *et al.* 1998).

Prevention and treatment of Diarrhea: Each year about 4 billion diarrhea episodes occur worldwide, accounting for almost 4% of all deaths and 5% of days lost in disability. Prevention of diarrhea is also a public-health issue. The effects of probiotics in the prevention and treatment of diarrhea are well studied (Lee and Salminen 2009).

Acute (Rotavirus) Diarrhea in children:

Most gastrointestinal infections affecting children in both developed and developing countries originate from rotavirus colonization of the gut (Van Niel 2002). Symptoms of infection by rotavirus manifest in gastroenteritis characterized by diarrhea and vomiting (Fric 2002). Currently, there is strong evidence indicating that *Lactobacillus casei (L. rhamnosus* L. GG) reduces the duration and severity of diarrhea associated with rotavirus in children (Fric 2002 and Gill and Guarner 2004) Supplementation with probiotic may

elicit a general immune response, as well as increased IgA (Immunoglobulin A) antibodies against rotavirus (Fric 2002).

Van Niel *et al.* (2002) conducted an analysis of nine clinical trials (n = 765) involving children younger than three years with acute infectious diarrhea who received *Lactobacillus* species, most frequently LGG. The meta-analysis revealed a reduced mean duration of diarrhea by 0.7 day (95% confidence interval [CI], 0.3–1.2 days) and a decrease in diarrhea frequency by a mean of 1.6 stools per day on day 2 of treatment (95% CI, 0.7–2.6 fewer stools) in children who received probiotics.

All probiotics are not equally effective in treating acute diarrhea in children. Canani *et al.* (2007) illustrated this point and emphasized that the particular probiotic preparation should be chosen based on solid efficacy data. In their study, 571 children age 3–36 months with acute diarrhea were randomized to one of six different treatment groups: oral rehydration solution alone (control group) or one of five probiotic preparations, which were administered for five days. Only two preparations—LGG and a mixture of four bacterial strains (*Lactobacillus delbrueckii* var *bulgaricus*, *Streptococcus thermophilus*, *Lactobacillus acidophilus*, and *Bifidobacterium bifidum*)—were associated with a significantly shorter median duration of diarrhea (78.5 and 70 hours, respectively; $p < 0.001$) compared with children who received oral rehydration solution alone (115.5 hours) One day after initiation of probiotics, children who were given LGG or the probiotic mixture also had a significantly lower daily stool output compared with the other groups ($p < 0.001$). The other three preparations (*S. boulardii*, *Bacillus clausii*, and *Enterococcus faecium* SF 68) did not significantly affect the duration and severity of diarrhea.

Antibiotic associated Diarrhea:

Decreases in the formation of colonic short-chain fatty acids and hyperosmolarity due to undigested carbohydrates are also thought to contribute to AAD (Seki *et al.* 2003). Probiotics may be efficacious in preventing AAD by stabilizing the microbial population of the colon by restoring resident flora and by stimulating the immune system. Stabilization of the microbial population will result in the production of short-chain fatty acids, specifically acetate, propionate, and butyrate, through fermentation of poly- and oligosaccharides, proteins, peptides, and glycoproteins (Macfarlane *et al.* 2003). Thus, their presence stimulates colonic water and sodium absorption, which allows for better stool formation (Tyagi *et al.* 2002). Additionally, short-chain fatty acids provide nutrition to the colonocytes, resulting in stimulated proliferation (Elsen *et al.* 1991). Few studies reported on AAD duration and severity as an outcome measure. Two studies *viz.* *Lactobacillus* GG (capsule) and *Saccharomyces boulardii* (Capsule) found that the duration and severity of AAD showed improvement after probiotic supplementation (Beniwal *et al.* 2003).

Clostridium difficile Associated Diarrhea:

Patients under treatment with antibiotics may develop *Clostridium difficile* colitis (Ogawa *et al.* 2001). It was reported that 80% of the patients responded well to the initial treatment of vancomycin or metronidazole. The remaining 20% may develop recurrent episodes of diarrhea, which may persist over several years, despite repeated antibiotic treatments (Ogawa *et al.* 2001, Ohashi *et al.* 2002).

S. boulardii in combination with oral vancomycin or metronidazole or both significantly decreased the recurrence of CDD. Other probiotics tested, including LGG and

Lactobacillus plantarum 299v in combination with oral vancomycin or metronidazole, were not effective in decreasing CDD recurrence rates. On the other hand, a recent study found that the rate of *C. difficile* colonization in 44 critically ill patients receiving antibiotics was significantly reduced by enteral administration of *L. plantarum* 299v ($p < 0.05$).Some studies have indicated that probiotics, especially *S. boulardii*, may prevent *C. difficile* overgrowth and decrease CDD recurrence.

Radiation-Induced Diarrhes: Radiation therapy disturbs the indigenous intestinal microbiota which may lead to acute enteritis and colitis (Sawada *et al.* 1990). Attempts to treat this complication with antibiotics, sucralfate and anti-inflammatory drugs have met with inconclusive results. Probiotics could be an alternative treatment. The above treatment with probiotics is potentially useful to protect cancer patients against the risk of radiation induced diarrhea (Lee and Salminen 2009).

Travelers' Diarrhea:

Most cases (80-85%) of traveler's diarrhea are due to bacterial pathogens, such as *Acromonas hydrophila*, *Campylobacter jejuni*, Enteroxigenic *E. coli*, Enteroaggregative *E.coli*, *Plesimonas shigelloides*, *Shigella* spp, *Salmonella* spp, *Vibrio cholera*, *Vibrio parahemoluticus* and *Yersinia enteocolitica* (Seow *et al.* 2002). Other less frequent causes of trveler's diarrhea are viruses (Norwalk or Rotavirus) and parasites (*Cyclospora, Crytosporidium, Entamoeba histolytica, Giardia lamblia*).

The efficacy of probiotics in the prevention of traveler's diarrhea appears to be species dependent, *L. rhamnosus, Saccharomyces boulardii* and a mixture of *Lactobacillus, Bifidobacterium* and *Streptococcus* showed positive effect, where as *L. acidophilus, L. fermentum* and *L. bulgaricus* showed no effect even at very high dose.

Diarrhea in Tube-Fed Patients:

Probiotics may not prevent ETF diarrhea; however, probiotics may reduce the number of days that patients suffer from diarrhea (Tsunoda *et al.* 2002).

Treatment of Irritable Bowel Syndrome: clinical symptoms of IBS include abdominal discomfort or pain, diarrhea, constipation, bloating and flatulence. The pathogensis of IBS remains unclear, but available evidence suggests that altered gut motility, visceral hypersensitivity and deregulation of the brain- gut axis are important mechanisms. An imbalance intestinal microbial profile (Kato *et al.* 2004) and enteric bacteria mediated mucosal inflammation (Tuomola *et al.* 1999) may be associated with IBS.

Some studies suggest that probiotics may be beneficial in reducing bloating and flatulence associated with IBS. A recent meta-analysis involving 20 trials (n = 1404) found that probiotics (most commonly lactobacilli and bifidobacteria) improved global IBS symptoms and reduced abdominal pain compared with placebo.

Prevention and treatment of Inflammatory Bowel Diseases: The chronic idiopathic inflammatory bowel diseases (IBD) such as Crohn's disease, ulcerative colitis and pouchits may be caused by a hyper-responsive cell-mediated immune response to intestinal commensal bacteria or microbiota aberrancies in genetically susceptible individuals(Charteris *et al.* 1998).

In case of Cohrn's disease, fewer patients relapsed after 6 months of combined treatment with *S. boulardii* and mesalazine (6.25%) compared with those taking mesalazine alone

(37.5%) (Guslandi *et al.* 2000). Trials examining the potential of other probiotics also found effectiveness in therapies that used *E. coli* Nissle or *Lactobacillus*. More specifically, a 12-month supplementation with *E. coli* Nissle 1917 strain at 5×10^{10} CFU reduced relapse in Crohn's disease patients compared with the placebo (30% vs. 70%, respectively) (Malchow 1997).

Pouchitis occurs when there is a nonspecific inflammation of the ileal pouch reservoir that is created surgically after an ileal-anal anastomosis. The etiology of pouchitis is unknown. It has been shown that remission produced by broad-spectrum antibiotics following surgery can be extended by the use of a mixture of eight bacteria (*L. casei*, *L. plantarum*, *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. longum*, *B. breve*, *B. infantis*, and *S. salivarius* subsp. *thermophilus*) given at a dose of 6 g/d providing 3×10^{12} CFU of viable lyophilized bacteria (Thompson 1986).

Treatment of Helicobacter pylori Infection: Several studies reported that certain probiotic bacteria exhibit inhibitory effect against *H. pylori* in vitro and in vivo (Charteris *et al.* 2001, Ohlson *et al.* 2002). Probiotic organisms do not appear to eradicate *H. pylori*, but they are able to reduce the bacterial load and inflammation in animal and human being. *L. casei* Shirota strain showed a significant reduction in the levels of H. pylori colonization in the antrum and body mucosa in vivo mouse model (Sgouras *et al.* 2004). *L. johnsonii* La1 and *L. gasseri* OLL2716 (Felley *et al.* 2001), *L. acidophilus* (Cats *et al.* 2003) were also reported to inhibit *H. pylori*.

Prevention of Postoperative Infections: Septic complications remain a major cause of morbidity and mortality in patients undergoing elective abdominal surgery despite the

routine use of antibiotic prophylaxis (Sullivan *et al.* 2003). It may be possible to reduce infection by correcting intestinal microbial imbalance by using combination of probiotics and prebiotics e.g. *Bifidobacteria breve* strain Yakult, *L. casei* strain Shirota etc. (Bonet *et al.* 2005).

Prevention and Treatment of Respiratory Tract Infections: Common virus respiratory infections such as common cold and influenza continue to casue considerable health hazards. Several probiotics microorganisms have been reported to enhance mucosal immunity against pathogen e.g. *Enterococcus faecalis*, *L. rhamnosus* GG etc (Jacobsen *et al.* 1999).

Prevention and Treatment of Allergic Diseases: Several studies have found that probiotics have beneficial effect on atopic eczema. There was a 50% reduction in the frequency of atopic eczema during the first two years of the children's lives in those given probiotics (e.g. *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb-12) compared with placebo (Lee and Salminen 2009).

Antitumor Effects: Antigenotoxicity, antimutagenicity and anticarcinogenicity are important potential functional properties of probiotics. There are numerous claims on the effects of probiotics binding to carcinogens and reducing the mutagenicity of urine and intestinal contents and their inhibitory properties on tumor in *in vitro* and in animal models. e.g. Superficial bladder cancer- L. casei Shirota, Breast cancer – Yogurt, Butter milk (probiotic strains not indicated) (Ouwehand *et al.* 2003).

Reduction of serum Cholesterol: Hypercholestrol is a risk factor for cardiovascular disease. It is suggested that intestinal Lactobacilli may reduce serum cholesterol level through bacterial assimilation in the intestine (Hawrelak *et al.* 2005) and deconjugation

of bile salts (Szajewska *et al.* 2007). Short-chain fatty acids produced by Lactobacilli may also inhibit hepatic cholesterol synthesis and distribution of cholesterol in the plasma and liver (Isolauri *et al.* 2000) e. g. *L. acidophilus* (Pohjavuori *et al.* 2004), *Enterococcus faecium* M-74 (Hatakka *et al.* 2001).

Enhancement of Vaccine Responses: The probiotics may improve response to some of the vaccines such as Haemophilus influenza, rotavirus and polio vaccines. E.g. *L. rhamnosus* GG, *L. acidophilus* CRL431 (Viljanen *et al.* 2005).

Applications of probiotics in livestock:

Probiotics were introduced in animal feed in the 1970s as supplements for the promotion of animal growth and improvement of their resistance to disease. Antibiotics have been used extensively in animal feeds since the 1950s to improve growth and feed conversion. The use of probiotics in farm animals is based upon the central principle that a healthy gut microbiota confers resistance to disease (Fuller 1992). Probiotics are useful in the treatment of disturbed intestinal microbiota and increased gut permeability.

Young animals are subjected to a multitude of stresses including transport, nutrition, temperature fluctuations and de-horning. Such stressors have been shown to induce imbalances of the host microbiota. Specific nutritional stresses include the change from milk to solid feed, from high fiber to high protein diets in young animals, and are associated with imbalances in the microbiota, which can lead to increased disease susceptibility, for example, increased incidences in diarrhea in post weaning piglets and ruminal acidosis in cattle. The use of probiotics in farm animals seeks to restore or

beneficially alter the microbiota present in young, stressed or antibiotic treated animals so that they can better resist infectious disease.

Following microorganism are used as probiotics for livestocks (Fuller 1992):

Bacillus species: *B. cereus, B. licheniformis, B. mesentericus, B. natto, B. toyoi*

Bifidobacterium species: *B. animalis, B. bifidum, B. breves, B. pseudolongum, B. subtile*

Lactobacillus species: *L. acidophilus, L. brevis, L. casei, L. delbrueckii* subsp. *Bulgaricus, L. fermentum, L. helveticus, L. plantarum, L. reuteri, L. rhamnosus.*

Other: *Aspegillus oryzae, Candida pintolopesii, Clostridium botulinum, Lactococcus lactis, Pediococcus pentosaceus, Saccharomyces cerevisiae*, Preemt-29species, cocktail of *E. coli* (17strains) and *Proteus mirabilis* (1 strain).

Bacterial probiotics are generally effective in chickens, pigs and preruminant calves, whereas fungal probiotics have shown better results in adult ruminants.

Poultry:

Probiotics have an indirect positive impact on the profitability of poultry production by improving their health such as improving immune system, modification of gut microbiota, reduce inflammatory reactions, decreased ammonia and urea excretion, lower serum cholesterol and increase mineral absorption (Teo and Tan 2007).

In 1973, Nurmi and Rantala discovered that newly hatched chicks could be protected from *Salmonella* infection by exposure to a suspension of gut contents from the adult chicken.

Bacillus subtilis, successful probiotic bacteria is used in poultry production. Apart from improving the growth of host, it is also known to inhibit the growth of pathogens in the digestive tract of chickens. The patented *B. subtilis* strain PB6 has the ability to kill

Clostridium perfringens, Campylobacter jejuni and *Streptococcus pneumonia* (Teo and Tan 2007, Lin *et al.* 2007). Spore forming *B. subtilis* can survive during pellets formation which is widely used for boiler feed production.

Protexin Boost (*L. plantarum, L. bulgaricus, L. acidophilus, L. rhamnosus, B. bifidum, S. thermopholus, E. faceium, A. oryzae, C. pintolopessi*): Boilers- significantly increased live weight gains at 2nd, 4th, 5th and 6th week, significantly increased carcass yield, breast, leg, bursa and spleen weight and antibody (Kabir *et al.* 2004).

Lactina (*S. thermophilus, E. faceium, Lactobacillus*): Male ducklings- improved feed conversion by 3.74% and reduced mortality after 93days. Significantly reduced the number of *E. coli*, *Lactobacillus* and *Salmonella* in cecum. No effects on blood parameters and serum cholesterol (Lee and Salminen 2009).

Lacto (*Lactobacillus* spp.): Layer- Significantly improved egg weight, size and mass. It also helps to increased passage rates of digesta, retentions of fat, calcium, phosphorus, copper and manganese (Nahashon *et al.* 1994).

Probiolac (*L. acidophilus, L. casei, B. bifidum, A. oryzae, S. faecium , Torulopsis* spp.): Layers- Significantly increased hen-housed egg production, shell weight and thickness, higher antibody (Panda *et al.* 2003).

Swine:

Pigs in the post weaning period are most vulnerable to enteric bacterial diseases and are therefore subject to extensive antibiotic treatments to reduce mortality and morbidity, the use of probiotics to improve growth performances and resistance against pathogenic bacterial diseases in pig has been widely studied. Combination of live Yeast and

Pediococcus acidilactici in piglet diets was found to improve the piglet's intestinal mucosa and help to develop immunity against pathogen (Lee and Salminen 2009).

Ruminants:

One of the earliest applications of probiotics was to treat ruminal acidosis by yeast. Apart from treating ruminal acidosis, fungus, yeasts and bacteria have been used in ruminants with varying success since the 1970s to increase milk production and weight gain, improve health status and resistance to diseases. Feeding calves with probiotics can reduce morbidity and mortality caused by intestinal and respiratory disease and this was confirmed by a recent report on veal calves treated with multi-species probiotic (Timmerman 2007).

Zhao *et al.* (1998) reported that colonization of *E. coli* O157:H7 in cattle can be eliminated by using a probiotic cocktail of 17 isolates of *E. coli* and one *Proteus mirabilis* strain. These were isolated from cattle on the basis of their ability to inhibit *E. coli* O157:H7 in vitro.

L. acidophilus: Calves- Significantly increased fecal Lacotbacilli count (Jonsson and Olsson 1985).

S. cerevisiae, A.oryzae extract: Cows- significantly reduced lactose and solids-nonfat contents (Higginbotham *et al.* 1994).

B. thermophilum, E. faecimum, L. acidophilus: Calves- improved feed conversion. Reduced diarrhea from 78-10% (Ishibashi and Yamazaki 2001).

L. acidphilus, L. lactis, B. subtilis (Biomate FG): Bull calves – Significantly increased mean capsular volume of blood after 10 days (Morril *et al.* 1995).

Rabbits:

Rabbits have been regularly used as test models for human probiotics (Myers 2007), mainly due to the similarity of digestive systems. As such, most of the studies concentrated on the ability of potential human probiotics to adhere and colonized the GI tract of rabbits.

B. cereus var. *toyoi*: Rabbits- Significant increase in final live weight by 2.5%, significant increase in daily weight gain by 4.2% and significantly improved feed conversion by 3.7%. Reduced morbidity by 43.4% (Thocino *et al.* 2005).

L. delbrueckii subsp. *bulgaricus, S. thermophilus, L. acidophilus*: Rabbit- Rabbits fed with *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* showed increased in intestinal *Enterococcus* population. Reduced *C. septicum* and *C. clostridiforme* from 80.7% and 12.7% respectively to zero. Significantly reduced plasma cholesterol.

L. casei Shirota : Infant rabbits- Decreased severity of diarrhea (Ogawa *et al.* 2001).

Pets:

The correlation between the gastrointestinal tract and overall health of the animals has led to a deluge of probiotics and prebiotics into pet food in recent years. Many such products contain mixtures of probiotic bacteria strains from *Pediococcus, Lactobcillus, Bifidobacterium, Bacillus, Streptocooccus, Enterococcus* or *Saccharamyces* yeast with claims of improved digestion, increased appetite, reduced diarrhea, increased firmness of stool, reduction in vomiting, reduction of body odor, reduction of flatulence or improved swallowing.

L. acidophilus NCC2628, NCC2766, *L. johnsonii* significantly reduced Inflammatory Bowel Disease Activity Index in all dogs suffering from food responsive diarrhea (Sauter *et al.* 2006).

Microbial Ecology of Human Gastro Intestinal Tract and the possible roles of probiotic organisms:

The concept of the gastrointestinal tract (GIT) as an ecosystem is based on the interactions among the resident assemblages of microorganisms, the structural and functional characteristics of the GIT, and the responses to dietary inputs. . The resident bacteria (the microbiome) are recognized to play a central role in GIT characteristics and host health (Martin *et al.* 2008, Tappenden and Deutsch 2007).

The resident bacteria also play an important role in host nutrition. The collective microfloras of humans, animals and fowl vary considerably with the architecture of their GI tract (Tannock 1995). Groups of microorganism are located at various locations throughout the GI tract and can reflect those that are either harmful or beneficial (Gibson and Roberfroid 1995). The digestive tract is composed of four major microbial population categories (Dubos et al. 1965, Fuller 1999, Tannock 1999).

Such as:

I. **Autochthonous microflora-** Populations of microbes that are present at high levels and permanently colonize the host. Molecular technologies have revealed that individual humans carry their own unique collection of autochthonous strains. However, disturbances to the GIT, such as antibiotic therapy, enteric infections or dietary stress can temporarily disrupt this group.

II. **Normal microflora-** Microorganisms that are frequently present, but can vary in number and be sporadically absent.

III. **True pathogens-** Microorganisms that are periodically acquired, but can persist, causing infection and disease.

IV. **Allochthonous microflora-** Microbes of another origin and present temporarily. Most probiotic cultures are allochthonous in nature. Of the predominant colonic microorganism, both *Lactobacillus* and *Bifidobacterium* species are considered exclusively beneficial and elicit no harmful or pathogenic responses within their normal ecological niches and they have positive or negative impact on autochthonous, normal and pathogenic microflora.

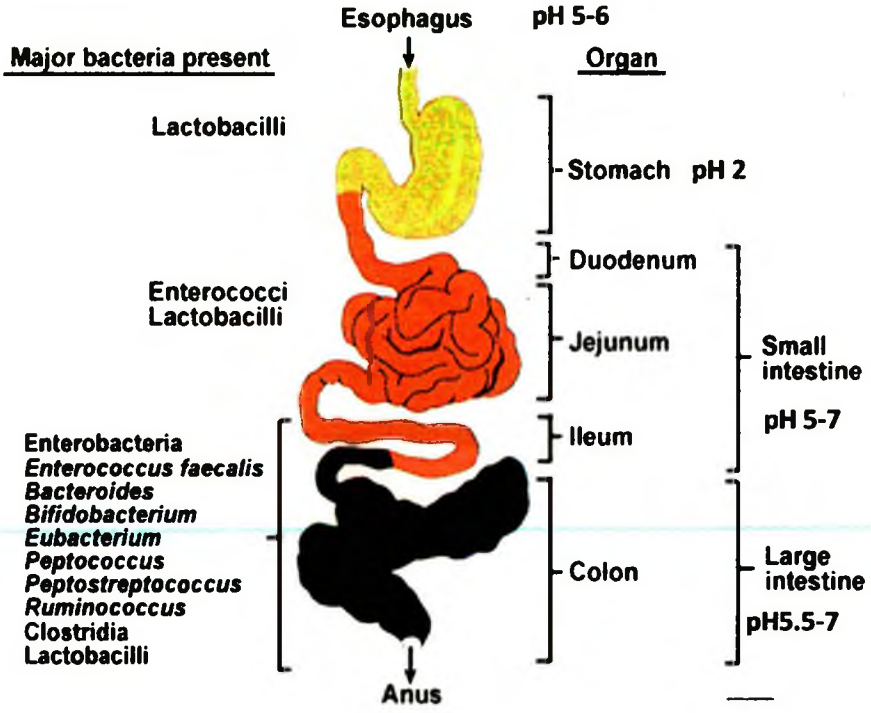

Figure 1: Bacteria distribution in the gastrointestinal tract.
(Source: www.agen.ufl.edu/~chyn/age2062/lect/lect, right panel adapted from Otte 2003)

Regulation of host homeostasis by probiotics

The concept of using probiotic microorganisms to prevent and treat a variety of human ailments has been around for more than 100 years. With the rise in number of multidrug resistant pathogens and the recognition of the role that the human microbiota plays in health and disease, a recent expansion in the interest in probiotics has been generated. Clinical efficacy of probiotics in adults, children, and infants has recently been shown for diseases associated with the gastrointestinal tract including inflammatory bowel diseases, diarrhea, irritable bowel syndrome, gluten intolerance, gastroenteritis, *Helicobacter pylori* infection, colon cancer, urogenital tract disorders, and allergy (Walker *et al.* 2006).

Workers suggested different mechanisms to explain how the probiotics can help the host. These mechanisms listed below (Rolfe 2000, Çakır 2003, Salminen *et al.* 1999).

1. Production of inhibitory substances: Production of some organic acids (e.g. lactic acid, acitic acid, propionic acid), hydrogen peroxide (e.g. *L crispatus* and *L. jensenii* in the female genital tract-inhibit gonococci), carbon dioxide (produced from heterolactic fermentation and inhibit the growth of many food spoilaging Gram negative psychrotrophic bacteria) and bacteriocins (e.g. nisin produced by several *Lactococcus lactis* strains which are inhibitory to both gram-positive and gram-negative bacteria.

2. Blocking of adhesion sites: Probiotics and pathogenic bacteria are in a competition. Probiotics inhibit the pathogens by adhering to the intestinal epithelial surfaces by blocking the adhesion sites. Several bacterial components, including cell wall proteins (e.g. *L. reuteri* 1063, *L. acidophilus* BG2FO4), carbohydrates (e.g. *L. fermentum* 104-R,

human *Lactobacillus* isolates), and lipoteichoic acids (e.g. *Bifidobacterium bifidum*, various *Lactobacillus* strains) are assumed to be involved in the adhesion of probiotics to intestinal contents.eg. Protein of act as adhesin

3. Competition for nutrients: Despite of the lack of studies in vivo, probiotics inhibit the pathogens by consuming the nutrients which pathogens need.

4. Stimulating of immunity: Stimulating of specific and nonspecific immunity may be one possible mechanism of probiotics to protect the host from intestinal disease. This mechanism is not well documented, but it is thought that specific cell wall components or cell layers may act as adjutants and increase humoral immune response.

5. Degradation of toxin receptor: Because of the degredation of toxin receptor on the intestinal mucosa, it was shown that *S. boulardii* protects the host against *C. difficile* intestinal disease.

Some other offered mechanisms are suppression of toxin production, reduction of gut pH, attenuation of virulence (Fooks *et al.* 1999).

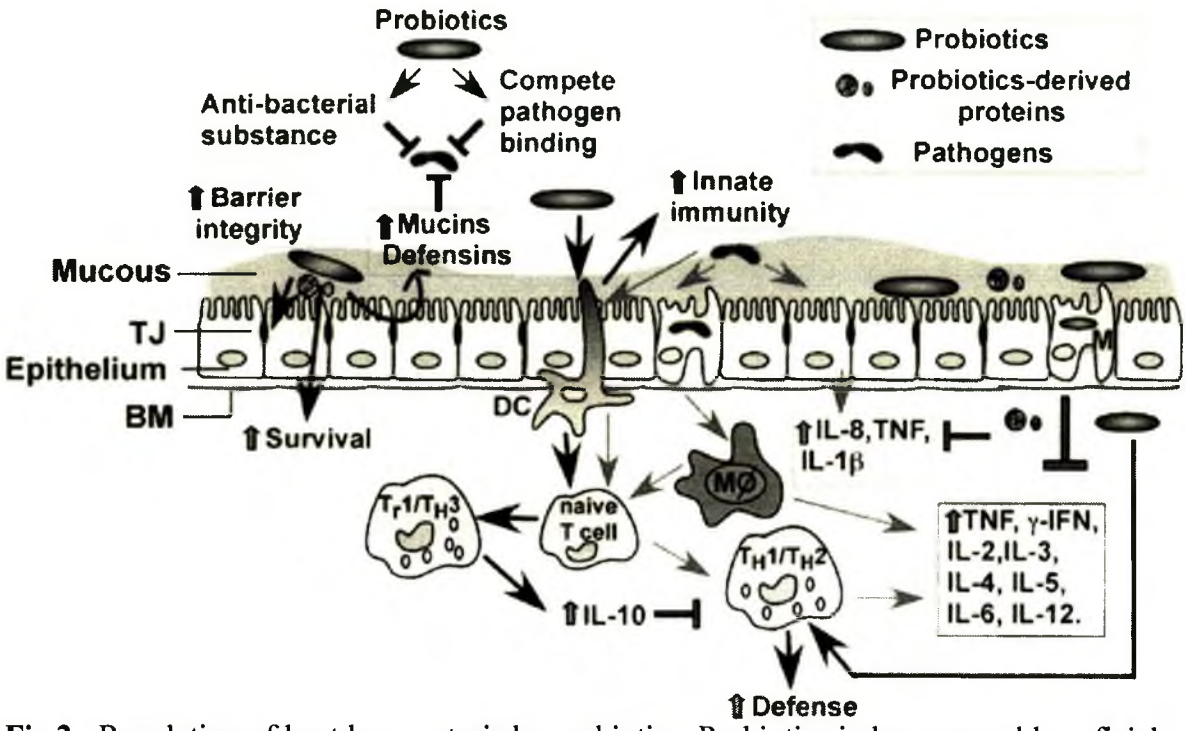

Fig.2 . Regulation of host homeostasis by probiotics. Probiotics induce several beneficial host responses. These include producing antibacterial substances, competing with pathogens for binding to epithelial cells, maintaining the intestinal microbial balance, promoting intestinal epithelial cell survival, barrier function, and cytoprotective responses, defining the balance between necessary and excessive defense immunity by increasing innate immunity, up-regulating anti-inflammatory cytokine production, and inhibiting pro-inflammatory cytokine production. BM: basement membrane; DC: dendritic cell; IL: interleukin; M: M-cell; TJ: tight junction.

Prebiotic:

Prebiotics were originally defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activities of one or a limited number of bacteria in the colon, thereby improving host health (Gibson and Roberfroid 1995). However, a more recent definition is that ''A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health''.

Common prebiotics in use include inulins, fructooligosaccharides (FOS), galacto-oligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides and lactulose. The majority of studies carried out to date have focused on inulin, FOS and GOS (Macfarland and Dublin 2008).

Plants containing inulin-type fructans primarily belong to the Liliales, e.g., leek, onion, garlic, and asparagus; or the Compositae, such as Jerusalem artichoke (Helianthus tuberosus), dahlia, and chicory (Cichorium intybus) and banana(Kunz *et al.*1999).

Xylo-oligosaccharides (XOS) are naturally present in fruits, vegetables, bamboo, honey and milk, and can be produced at industrial scale by enzymatic hydrolysis from xylan, which is the major component of plant hemicelluloses and therefore readily available in nature (Alonso *et al.* 2003, Va´zquez *et al.* 2000). XOS are non-digestible carbohydrates and have been suggested to exert prebiotic activity.

Resistant starches are defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Asp 1997). There are four main groups of resistant starches: RS1, RS2, RS3 and RS4. RS1 is physically inaccessible starch (i.e., starch in whole grains), RS2 is granular starch i.e., starch in green bananas), RS3 is retrograded starch (i.e., starch in cooked and cooled potatoes) and RS4 is a chemically-modified starch (i.e., an esterified starch).

fructooligosaccharides are complex carbohydrates found. Four major classes of structurally different fructans can be distinguished: inulin, levan, mixed levan, and neoseries. Fructans of various types can be found as natural components in honey, fruits and vegetables such as Jerusalem artichoke, banana, chicory, onion, leek, garlic, rye, barley and salsify.

Synbiotic:

Probiotics are not the only functional food ingredients developed to improve human health by modulating the intestinal microbiota. Prebiotic ingredients represent an alternative and potentially synergistic approach. These non-digestible carbohydrates pass though to the colon where thy selectively stimulate the proliferation and or activity of beneficial microorganism within the intestinal microbiota (Gibson and Roberfroid 1995). Ingredients and foods that contain both prebiotic and probiotics are called synbotics.

Aims and objectives

The aims of the studies are:

1. Isolation of probiotic microorganisms from different fermented milk products and vegetable available in Bangladesh
2. Characterization (morphological, physiological and biochemical) of the selected isolates.
3. Provisional identification of the selected organisms.
4. To study the possible interactions of the isolates with randomly selected pathogenic microorganisms.
5. Fermentation experiment to study the possible variation in the nutritional factors in some common fruits and vegetable.

Materials and Methods

Sources of probiotic microorganisms:

For isolation of probiotic organisms different types of fermented vegetables and fermented milk products were used which are listed in the following table

Table 6: Samples collected from the retailers of the following areas:

Fermented milk product:	
Sample	**Collection area**
Yoghurt Sour Sweet	Dhaka, Bikrompur, Bogura, Jessore
Cheese Cow's milk salted Cow's milk Less salted Buffalo's milk	Dhaka New Market , Dhaka Baitul Mokarm Market
Fermented cream	Milk was heated at low temperature to produce thin film over the milk, these milk film were collected and kept at room temperature for several days for fermentation.
Whey	Local market place- Dhaka Newmarket, Niltkhet

Fermented vegetable product:	
Sample	**Commercial name of fermented product**
Cabbage	Sauerkraut
Cucumber	Cucumber pickle

Fermented juice:	
Sample	**Collected area**
Juice from Date plam tree	Jessore, Dhaka

Media for isolation:

For the isolation of probiotic microorganisms several selective media were used. MRS (Corry *et al.* 2003) medium is the generalized medium for lactic acid bacteria isolation (Sneath *et al.* 1989). During this work modified MRS was used to enhance growth of the probiotic microorganisms. Selective media such as Leuconostoc medium (Benkerroum *et. al* 1992), Bifidobacteria medium (Nebra and blanch 1999) and Yeast extract-malt extract medium (Wickerham 1951) were used. In addition to above media, Lee's medium (Atlas 1997) and Lactose mineral agar (ATCC) medium were also used for the isolation of Lactobacilli.

Isolation and purification procedure:

Since the isolation was attempted in anaerobic and aerobic environment, after incubation for 3-5 days at 30°C temperature, colonies with different morphology were isolated and purified by restreaking on the same medium.

Procedure for maintaining anaerobic condition:

Several methods were followed to provide anaerobic condition for growing microorganisms.

I. Use of Sodium thio Glycholate: As probiotic organisms are anaerobic or microaerophilic in nature, 0.2% sodium thioglycolate was used in the media as oxygen scavenger (Brashears *et al.* 1998). Sodium thioglycholate is an active reducing agent; it works effectively in the closed system where oxygen can not

be replaced once it is consumed. Because of its autooxydation nature, it easily consumes oxygen from the medium and allows anaerobic or microaerophilic microorganisms to grow.

II. Cystine: This amino acid contain sulfhydryl groups and can work as oxygen scavenger from growth medium (Atlas *et al.* 1995)

III. Use of liquid Paraffin: Liquid Paraffin prevents microbial growth to come in contact with free oxygen molecule in environment. After inoculation of microorganisms in broth or solid media, paraffin was poured over the media to cover the surface.

IV. Use of alkaline Pyrogallate: KOH crystals and Pyrogallol (1, 2, 3 tri hydroxybenzene) crystals mixture were used in the anaerobic jar for the absorption of oxygen and production of CO_2 (SAB 1957).

V. Use of Gas pack: This method is based on hydrogen generation in jar. It can react with available O_2 to form water. Palladium pellets catalyses the reaction at room temperature.

VI. Candle jar method was also applied as described by Dubey and Maheshwary 2002.

For the isolation of anaerobic organisms, culture plates were placed in anaerobic jar and kept at 30°C for 3-4 days.

pH of media:

For isolation purpose, pH of the media was maintained between 6.0-6.8.

Antibiotics:

Antibiotics were used to maintain selectivity. In this work, Vancomycin and Tetracycline were used by following the method of Benkerroum *et al.* 1992.

Vitamins:

To enhance selective microbial growth different vitamin sources were used. Nutritionally Probiotic microorganisms are fastidious and many vitamins, amino acids, purines and pyrimidines must be supplied because of their limited biosyntehetic capabilities (Prescott *et al.* 2005). In the isolation media, Riboflavin (1mg/l) and Thiamine HCl (1mg/L) were used as described by Nebra and blanch (1999).

Screening of microorganisms:

Cell morphology, Gram staining, spore staining and catalase tests were performed for preliminary screening of probiotic microorganisms. Several parameters were also studied to select probiotic microorganisms for further applications.

Subculture and maintenance:

As most of the microorganisms were microaerophilic and anaerobic in nature, they were subcultured in modified MRS broth medium and TS medium by adding 0.2% Sodium thioglycholate as oxygen scavenger. For the microorganisms which were isolated in anaerobic condition, paraffin was used over the media and kept in the anaerobic jar for 4-5 days.

The organisms were kept in refrigerator for further studies.

Colonial Morphology of Isolates:

The bacterial colonies were grown on the isolation media (e.g. MRS, Bifidobacterial medium, Leuconostoc medium etc) to observe their colony characteristics. To study elaborately, isolated colonies were observed under microscope and photographs were also taken.

Microscopic studies of the selected bacterial strains:

The bacterial cells were examined under microscope (Nikon-Optiphot) and photomicrographs (Nikon, FX35WA, Japan) were taken. Several techniques including wet mount, differential staining (Gram staining, Spore staining) were used.

Growth curve determination:

For growth curve determination, isolated microorganisms were cultured in modified MRS medium at 30°C and the pH of the medium was 6.8.

Growths were observed after 8hrs, 14hrs, 20hrs, 24hrs, 28hrs, 32hrs, 36hrs and 40hrs and at the same time, pH of the culture medium was also recorded. This test was performed in duplicate. Growth of microbes was measured spectrophotometrically absorbance at 610nm (Figure 29-34).

Growth Response at Different pH (Sneath *et al.* 1986):

For testing the growth response of the isolates at different pH (4.0, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5), buffered modified MRS broth (in 4:1 ratio) was adjusted to a range of pH i.e. 4.5, 6.5 and 8.5. Buffer solutions were used by following compositions as described by Williams *et al.* 1971.

Growth was measured after 48hrs incubation by using spectrophotometer at 610nm (absorbance) (Table 19-20 and Figure 25-28).

Growth at different temperature:

To observe the effect of temperature on the selected microorganisms, there were cultured in modified MRS broth medium. The broth tubes were inoculated in duplicates with freshly bacterial culture and were allowed to grow at different temperatures *viz.* 4°, 10°, 15°, 25°, 37°, 45°, 50° and 55°C for 48 hours. Results were estimated by spectrophotometer at 610 nm. (Table17-18 and figure 21-24)

Oxygen requirement test (SAB 1957):

To observe the relationship between microorganisms and free oxygen, deep glucose agar test was performed. In the deep glucose agar test, the oxygen content of the medium decreased with depth until the medium becomes anaerobic toward the bottom of the tube.

Bile salt tolerance (Tambekar and Bhutada 2010):

After ingestion of probiotic microorganisms, they should go through the adverse condition of GIT where low pH and high concentration of bile salt are encountered. To demonstrate

the chance of survivability in the small intestine and colon, the organisms were tested for their tolerance to bile salts. For this purpose, modified MRS medium supplemented with 0.5%, 1% and 2.0% (Tambekar and Bhutada 2010) were used.

For this test, comparative growth of the isolated microorganisms were observed on the MRS medium supplemented with 0.5, 1% and 2.0% bile salt and MRS medium as control. The results are presented in the table (11-12).

Hemolysis test (Atlas *et al.* 1995):

For the screening of probiotic microorganisms, hemolysis test was coducted and test organisms were cultured by point inoculation, on the blood agar medium which was supplemented with 5% sheep blood. Inoculated cultures were incubated at 37°C for 48 hrs.

Biochemical studies:

During this research work a number of biochemical tests were performed to characterize the isolates and for their provisional identification. The standard methods were followed to test for Catalase (Clause 1995), Oxidase (Collins and Lyne 1984), KOH test (Halebian *et al.* 1981), Decomposition of urea (Biolife 1999), Degradation of Tyrosin (Sneath *et al.* 1986), hydrolysis of Esculine (Collins and Lyne 1984), Casein (Collins and Lyne 1984), Starch (Claus 1995), Hydrogen Sulfied production, VP and MR test (Bryan 1950), Nitrate reduction test (SAB 1957), Indole production (Biolife 1999), utilization of Propionate (Sneath *et al.* 1986), Arginine dihydrolase test (Schaad 1988) and determination of Phenylalanine and Egg Yolk lecithinase test (Sneath *et al.* 1986). For the test of utilization of citrate, two types of media were used *viz.* Simmon's citrate agar medium and Tomato

juice lactate agar medium as described by Claus (1995) and Harigan and McCance (1976). (Table 11-12)

Action on litmus milk:

The test was performed in duplicate. Result of the test was recorded and presented as per following plane (Harigan and McCance 1976)

REACTION	LITMUS COLOUR	NOTE
Acid production	Pink	pH become 4.5. sufficient acid production cause milk clot-**Acid Clot**
Gas production	-	Bubble or fissures will visible in the medium.
Reduction of litmus	White	White starts in tube bottom
Coagulation of casein protein	Litmus colour remaining blue	Protiolytic activity affecting the casein-**Sweet clot**
Hydrolysis of casein protein (partial or nearly complete hydrolysis)	Clearing and loss of opacity in the milk medium. In case of partial hydrolysis, it will found at the top in tube.	**Peptonisation**

Fermentation test:

In this study the fermentation tests of the following carbohydrates were done.

Monosaccharide:

Pentose- Xylose

Hexose- Glucose (dextrose), fructose (levulose), Galactose, Mannose, Rhamnose, sorbose

Sugar Alcohols: Sorbitol, glycerol, Inositol, Mannitol, Dulcitol, Adonitol

Disaccharide: Sucrose, Maltose, Lactose, Cellobiose, Trehalose, Melibiose

Polysaccharide: Starch, Inulin

MRS medium for fermentation (de Man, Rogosa and Sharpe 1960) supplemented with 1% carbohydrate source was used for fermentation test. Bromocresol purple was added as pH indicator. After inoculation, tests were incubated at 30°C for 48 hrs.

Results

Isolation of microorganisms: More than 100 microbes were isolated from fermented food samples by using different types of media. Most of the microbes were isolated in modified MRS medium. While for the isolation of *Bifidobactetria* (Nebra and blanch 1999) and *Leuconostoc*, specialized media were used (Benkerroum et. al 1992).

Large number of microorganisms including bacteria and yeast were isolated from fermented milk products such as yogurt and cheese. From fermented vegetable (eg. Sauerkraut, Gherkin) also a few bacteria were isolated. Date's juice was also found to be a rich source of probiotic microorganisms.

From aerobic culture 13 bacteria were selected for further studies. Among them 6 isolates were from milk products, 5 isolates from Date's juice and 2 isolates from fermented vegetables.

From anaerobic culture, a total of 30 bacteria were selected. Among them 20 isolates from milk products and 10 isolates were from fermented vegetables.

Colony characteristics of selected isolates: Most of the colonies were circular, moist and opaque. Isolated colonies on Bifidobacterial medium showed bluish green colour. (Fig 3-8 and Table 7-8)

Microscopic observation and staining properties: Among 43 isolates, 7 isolates were coccus and the rest were rod shaped. Vegetative cells were non motile and Gram- positive. All the isolates were non spore former. (Fig-9-19 and Table-9-10)

Hydrolysis of Starch, Casein, Arginine and Esculine: None of the strains could hydrolysis starch. Among the 43 isolates, 33 hydrolyzed casein. Anaerobic isolates C1/7a,

C1/10 and Cab1 and aerobic isolates DJJ1, DJJ4, DJJ3, FC2, CAB1, DJD4 and TBY5 did not have casease enzyme. 14 Anaerobic bacteria and 5 aerobic bacteria showed positive reaction in arginine hydrolysis test. Except 14 isolates, all bacteria hydrolyzed esculine.(Figure-20 and Table 11-12)

The result of VP, MR, Hydrogen sulfide and Indole production: Selected 43 probiotic microorganisms did not produce indole and hydrogen sulfide. (Table 11-12)

Action on litmus milk: In litmus milk test, all isolates produced acid except anaerobic C1/10 and aerobic DJJ3 and DJD4. Anaerobic isolates C1/10 and Cab1 did not form acid clot and 7 aerobic isolates formed acid clot. Among all isolates only aerobic isolate FC2 formed sweet clot. 14 anaerobic isolates and 4 aerobic isolates produced gas in the reaction. Among 43 isolates 33 organisms were casein positive and all were citrate negative (Table15).

Test for decomposition of Urea, Tyrosine and Nitrate reduction: Isolated all probiotic microorganisms were tyrosine negative. Three aerobic bacteria (C3/4, C3/2, C3/1b) and two anaerobic bacteria (C1/5a and Cab6) were able to decomposition of urea i.e. they have urease enzyme. Among 43 isolates, 6 bacteria (C3/4, C3/2, C1/1, Cab 1, Cab 2 and Cab 5) and 4 bacteria (C3/4, C3/2, C3/1b and C1/3) respectively anaerobic and aerobic bacteria were nitrate positive (Table 11-12).

Production of Lecithiniase, Protease, Catalase and Oxidase: Selected 43 isolates were lecithinase and oxidase negative. Among 43 isolates, 14 bacteria (5 aerobic and 9 anaerobic) were able to produce protiolytic enzyme in egg yolk licithinase medium. Except 3 aerobic

isolates (C3/4, C3/2 and C3/1b), all aerobic and anaerobic bacteria were catalase negative (table 11-12).

Utilization of Citrate, propionate, Phenyl alanine dtermination, KOH and Deep glucose agar test: All isolates showed negative result in citrate utilization, phenyl alanine determination and propionate test. In presence of KOH, bacterial suspension did not become thick and stringy which indicate all isolates were Gram (+) ve. Among the aerobic isolates, 6 isolates were facultative anaerobe, 3 aerotolerant anaerobe, 2 obligate aerobe and only one was microaerophilic in nature. Most of the anaerobic isolates were aerotolerant anaerobic except C1/14 and Cab3 were microaerophilic (Table 16).

Hemolytic activity: All aerobic and anaerobic isolates were β hemolysis and α hemolysis negative. Thirteen anaerobic and five aerobic microorganisms were γ hemolysis positive i.e. they can grow in presence of blood (Table 11-12).

Acid production from different carbohydrate sources: Twenty-four types of carbohydrates of different groups e.g. Monosaccharide, Disaccharide, Polysaccharide and Sugar alcohol, were used for fermentation test. All aerobic microbes produced acid in presence of Lactose. But in presence of Adonitol, Inositol, Melibiose, Glutamate and Starch, no one could able to produce acid. All anaerobic isolates produced acid from Glucose, Fructose and Lactose (Figure20 and Table 13-14).

Growth response in presence of Bile salt: Selected isolates were tested to observe their growth in presence of bile salt in the medium. Five aerobic and eight anaerobic isolates showed better growth then the control medium and rest had no effect on their growth (Table11-12).

Growth response at different pH: Selected isolates were tested for their growth at different pH level. Growths of aerobic microbes were tested at pH 4.0, 4.5, 5.5, 6.5, 7.5 and 8.5 and in case of anaerobic microbes tested pH level were 3.5, 4.5, 5.5, 6.5, 7.5 and 8.5. (Table 19-20 and Figure 25-28)

Growth response at different temperature: The growth of isolated probiotic microorganisms were observed at different temperatures. In case of aerobic bacteria, growth observed at 5^{o}, 10^{o}, 25^{o}, 30^{o}, 37^{o}, 45^{o} and 50^{o}C and for anaerobic microorganisms temperatures were 5^{o}, 10^{o}, 15^{o},25^{o}, 37^{o}, 45^{o} and 50^{o}C. (Table17-18 and Figure 21-24)

Growth curve determination of isolates: Among the total isolates, 13 aerobic bacteria and 17 anaerobic bacteria were selected to observe their growth at different times and at the same time their pH also recorded. In case of aerobic bacteria, growth increased gradually from 18hrs to 22 hrs and after 30hrs pH of the medium decreased gradually. (Figure 29-34)

Provisional identification of the isolates: With the available facilities, the isolates were provisionally identified with the help of Bergey's Manual of Systematic Bacteriology Vol.2 ed. 9^{th} (Sneath et al. 1986). (Table 21)

Table 7: Colony characteristics of aerobic bacteria:

Isolates	Form	Elevation	Margin	Surface	Colour and Optical characteristics	Diameter (mm)
C3/4	Circular	Convex	Entire	Smooth glossy	Opaque Cream	5.1
C3/2	Circular	Umbonate	Entire	Smooth glossy	Opaque Cream	5.3
C3/1b	Punctiform	Convex	Wavy	Smooth glossy	Opaque Blossom	1.3
C1/3	Circular	Raised	Entire	Smooth	Opaque Off white	4.9
DJJ1	Punctiform	Raised	Entire	Smooth	Opaque Off white	0.4
DJJ4	Punctiform	Raised	Entire	Smooth	Opaque Off white	0.4
DJD1	Circular	Convex	Entire	Smooth	Opaque Cream	1.1
DJJ3	Punctiform	Raised	Entire	Smooth	Opaque Off white	0.5
FC2	Punctiform	Raised	Entire	Smooth	Opaque Off white	0.5
CAB1	Circular	Convex	Entire	Smooth	Opaque Cream	1.3
CAB3	Punctiform	Pulvinate	Entire	Smooth	Opaque Off white	0.9
DJD4	Punctiform	Raised	Entire	Smooth	Opaque Off white	0.7
TBY5	Punctiform	Raised	Entire	Smooth	Opaque Off white	0.3

All organisms grown in MRS medium except DJJ1, DJD4, FC2 and TBY5, which were isolated on Leuconostoc medium.

Table 8: Colony characteristics of anaerobic bacteria (Cont.):

Isolates	Form	Elevation	Margin	Surface	Colour and Optical characteristics	Diameter (mm)
C1/7a	Punctiform	Raised	Irregular	Smooth glossy	Opaque Apple white	0.6
C1/5a	Circular	Flat	Erose	Smooth glossy	Opaque Lime white	1.2
C1/7	Circular	Umbonate	Entire	Smooth glossy	Opaque Off white	2.0
C1/14	Circular	Convex	Irregular	Smooth glossy	Opaque Dark bluish green	1.8
C1/12	Circular	Raised	Wavy	Smooth glossy	Opaque Greenish white	1.1
C1/8(1)	More or less circular	Flat	Irregularly entire	Smooth glossy	Opaque Milk white	2.1
C1/12a	Circular	Convex	Undulater	Smooth glossy	Opaque Cream	1.2
C1/12s	Punctiform	Concave/ umbonate	Entire	Smooth matt	Opaque Brown	0.8
C1/10	Circular	Raised	Entire	Smooth glossy	Opaque Off white	1.6
C1/12b	Circular	Convex	Entire	Smooth glossy	Opaque Yellowish white	2.6
C1/16	Circular	Raised	Entire	Smooth glossy	Opaque Greenish white	1.4
DY(w)	Irregular	Flat	Filamentous	Smooth glossy	Opaque White	1.4
DY(w)3	Circular	Raised	Entire	Smooth glossy	Coral green	1.6
C1/2	More or less circular	Raised	Wavy	Smooth glossy	Opaque Off white	1.2
C2/2	Punctiform	Raised	Erose	Rough glossy	Opaque White	0.8

All organisms grown in MRS medium except C1/14 which was isolated on Bifidobacterial medium

Table 8: Colony characteristics of anaerobic bacteria

Isolates	Form	Elevation	Margin	Surface	Colour and optical characteristics	Diameter (mm)
C1/4	Circular	Flat	Undulate	Smooth matt	Opaque Off white	2.8
GRK5	Irregular shape	Flat	Entire	Smooth glossy	Opaque Light brown	1.0
GRK6	More or less circular	Convex	Wavy	Smooth glossy	Opaque Light brown	1.1
GRK7	More or less circular	Flat	Irregularly wavy	Smooth glossy	Opaque Off white	1.2
C1/8	Irregular	Flat	Undulate	Smooth glossy	Opaque Off white	2.7
C1/1	Circular	Raised	Filamentous	Rough glossy	Opaque Cream	2.9
C1/1b	Circular	Convex	Entire	Smooth glossy	Opaque Brilliant white	2.4
C2/6	Circular	Convex	Entire	Smooth glossy	Opaque Salsa	1.7
Cab1	Circular	Flat	Entire	Smooth glossy	Opaque Cream	1.2
Cab2	Punctiform	Raised	Wavy	Smooth glossy	Opaque Cream	0.6
Cab3	Circular	Umbonate	Undulate	Smooth glossy	Bluish green	2.6
Cab4	Circular	Umbonate	Entire	Smooth glossy	Opaque Light coffee	3.5
Cab5	Circular	Convex	Entire	Smooth glossy	Opaque Butter white	1.9
Cab6	Punctiform	Flat	Entire	Smooth glossy	Opaque Light coffee	0.9
Cab8	Circular	Raised	Entire	Smooth glossy	Opaque Chamois	2.1

All organisms grown in MRS medium except Cab 3 which was isolated on Bifidobacterial medium

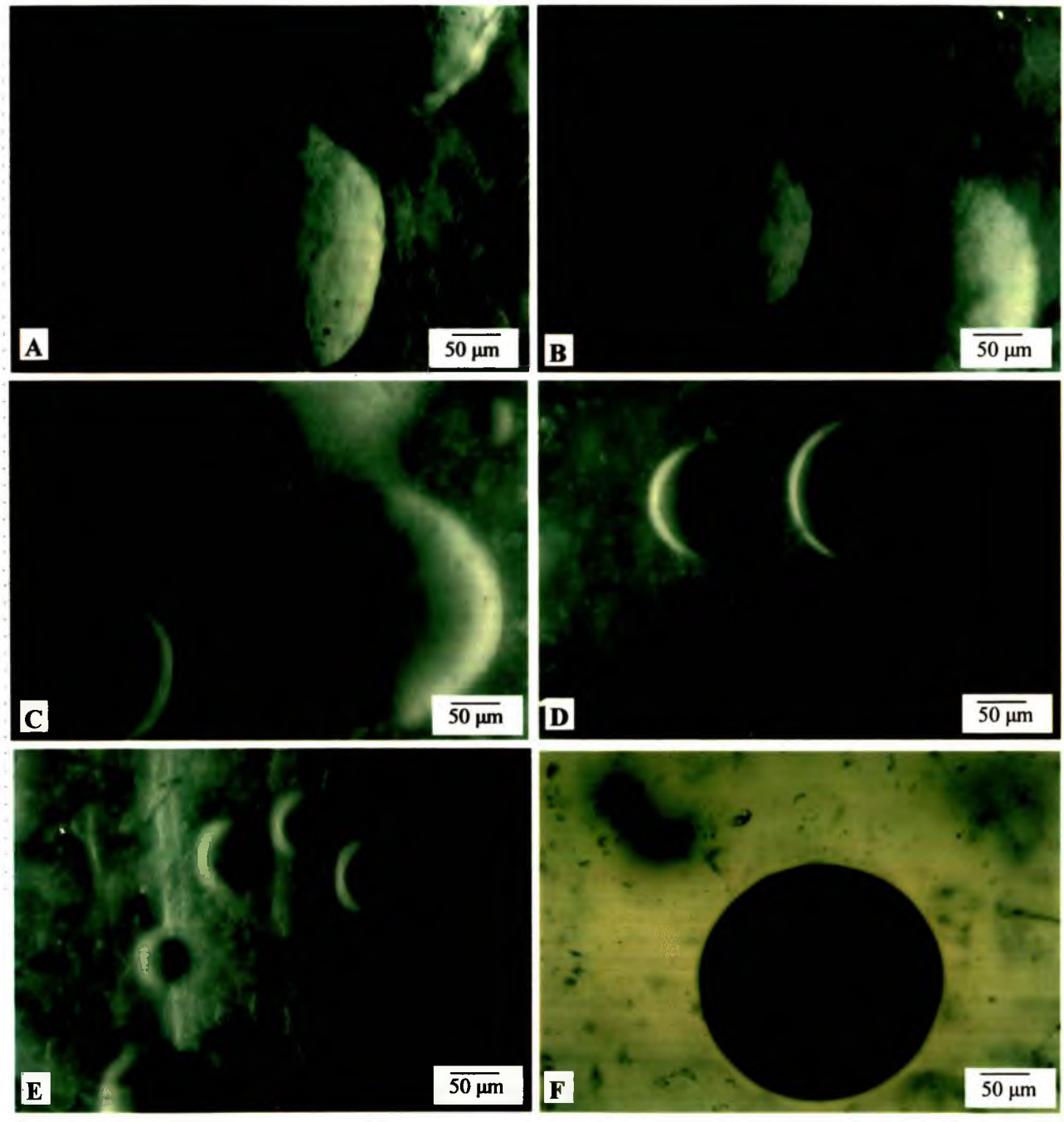

Fig 3. Colonies of C3/2 (A), C3/1b (B), C1/3 (C), DJJ1 (D), DJJ4 (E) and DJD1 (F) as observed under microscope (Bar = 50µm).

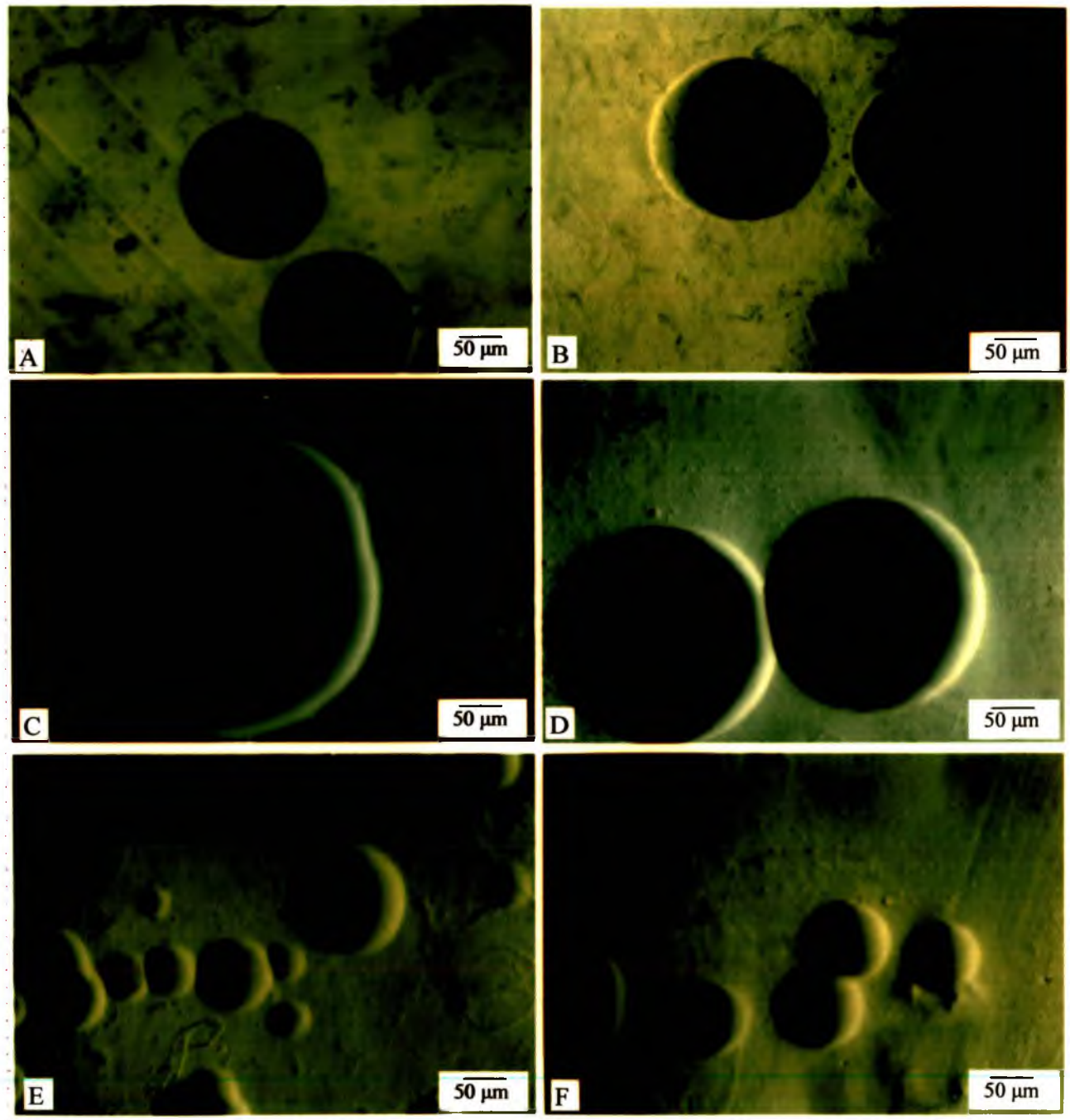

Fig .4. Colonies of the selected isolates as observed under microscope **A.** DJJ3, **B.** FC2, **C.** CAB1, **D.** CAB3, **E.** DJD4 and **F.** TBY5 (Bar = 50µm).

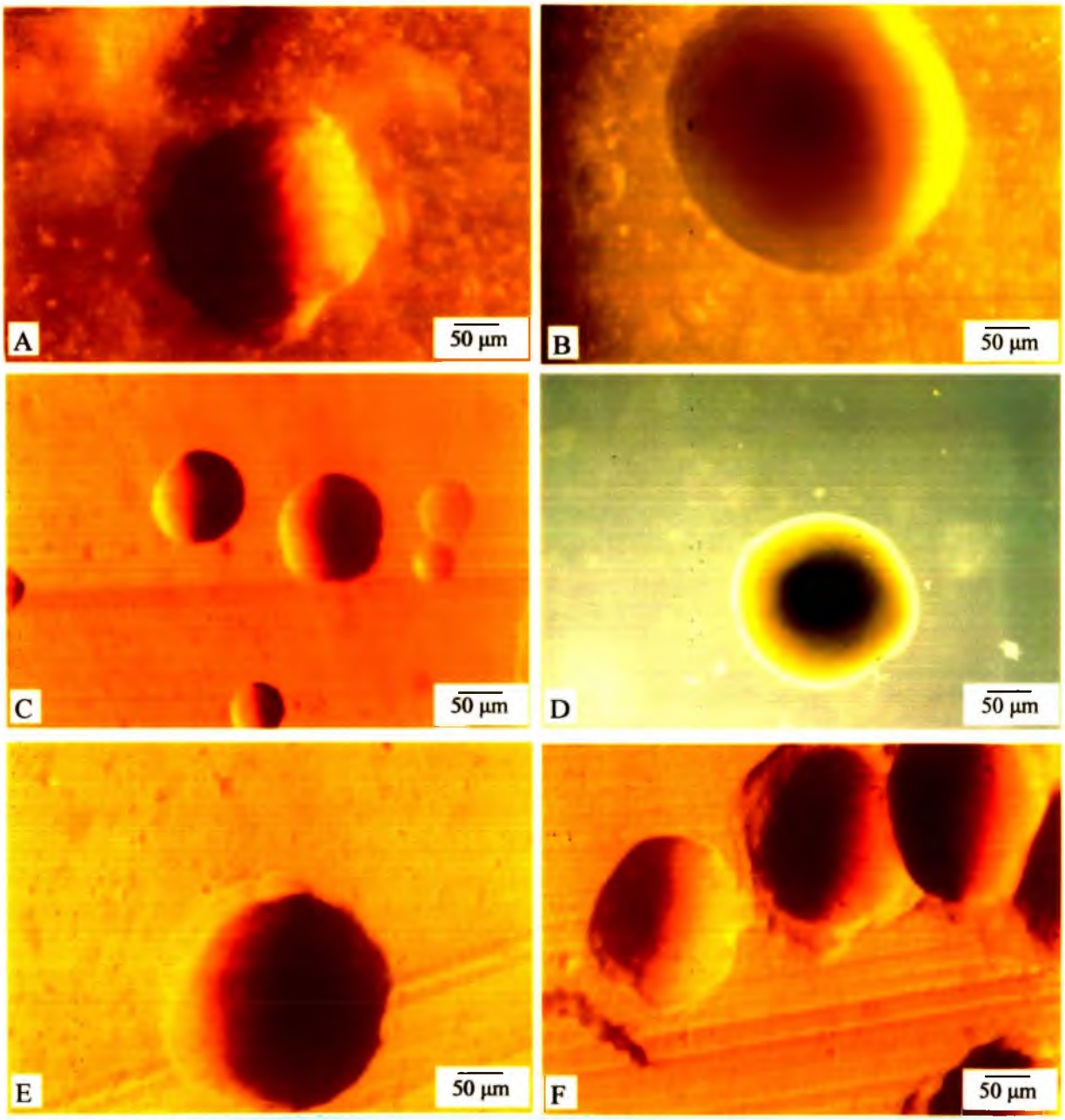

Fig.5.Colonies of the selected isolates as observed under microscope **A.** C1/7a, **B.** C1/5a, **C.** C1/7, **D.** C1/14, **E.** C1/12 and **F.** C1/8(1) (Bar = 50µm).

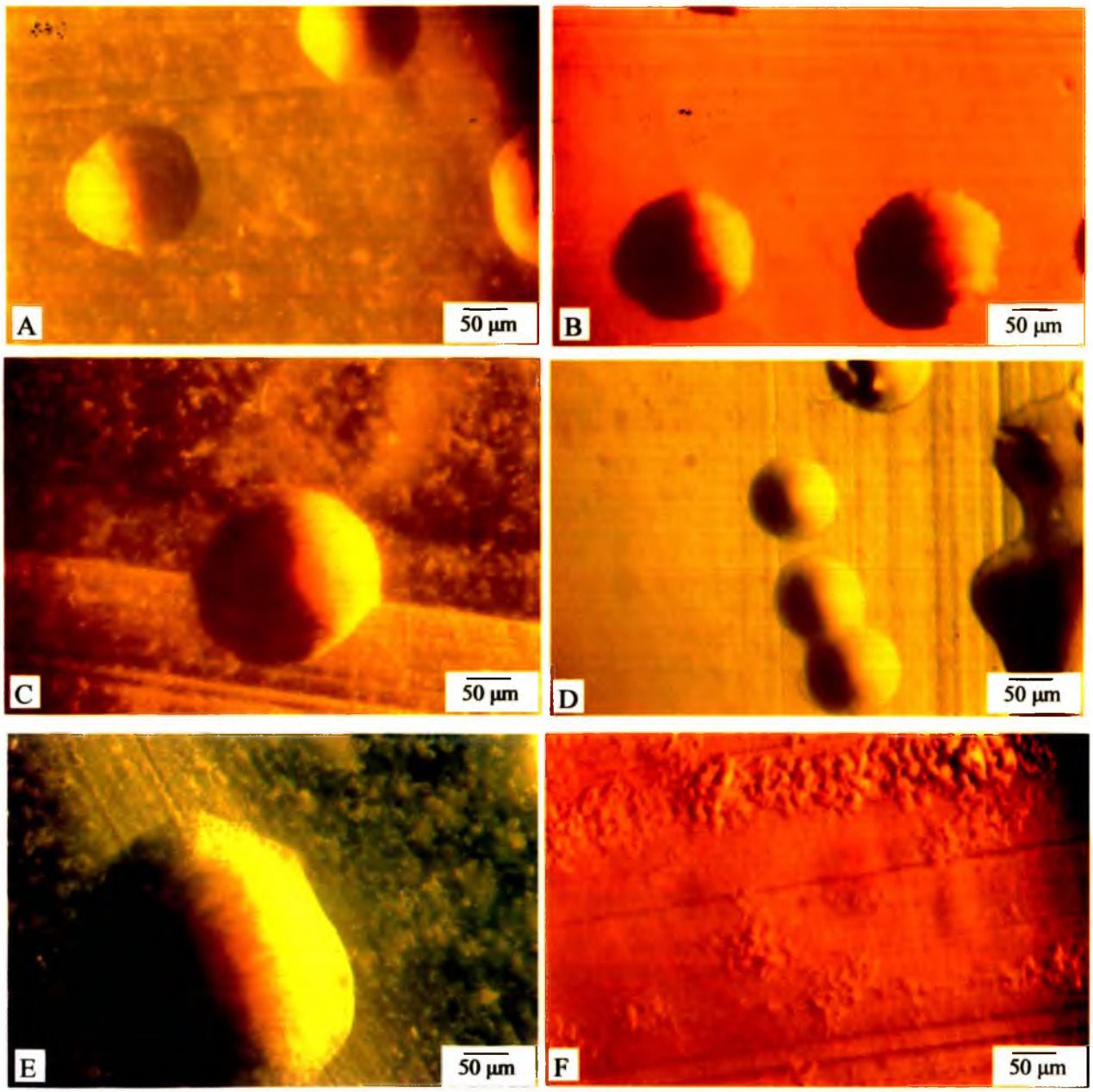

Fig. 6. Colonies of the selected isolates as observed under microscope **A.** C1/12s, **B.** C1/12a, **C.** C1/10, **D.** C1/12b, **E.** C1/16 and **F.** DY (w) (Bar = 50µm).

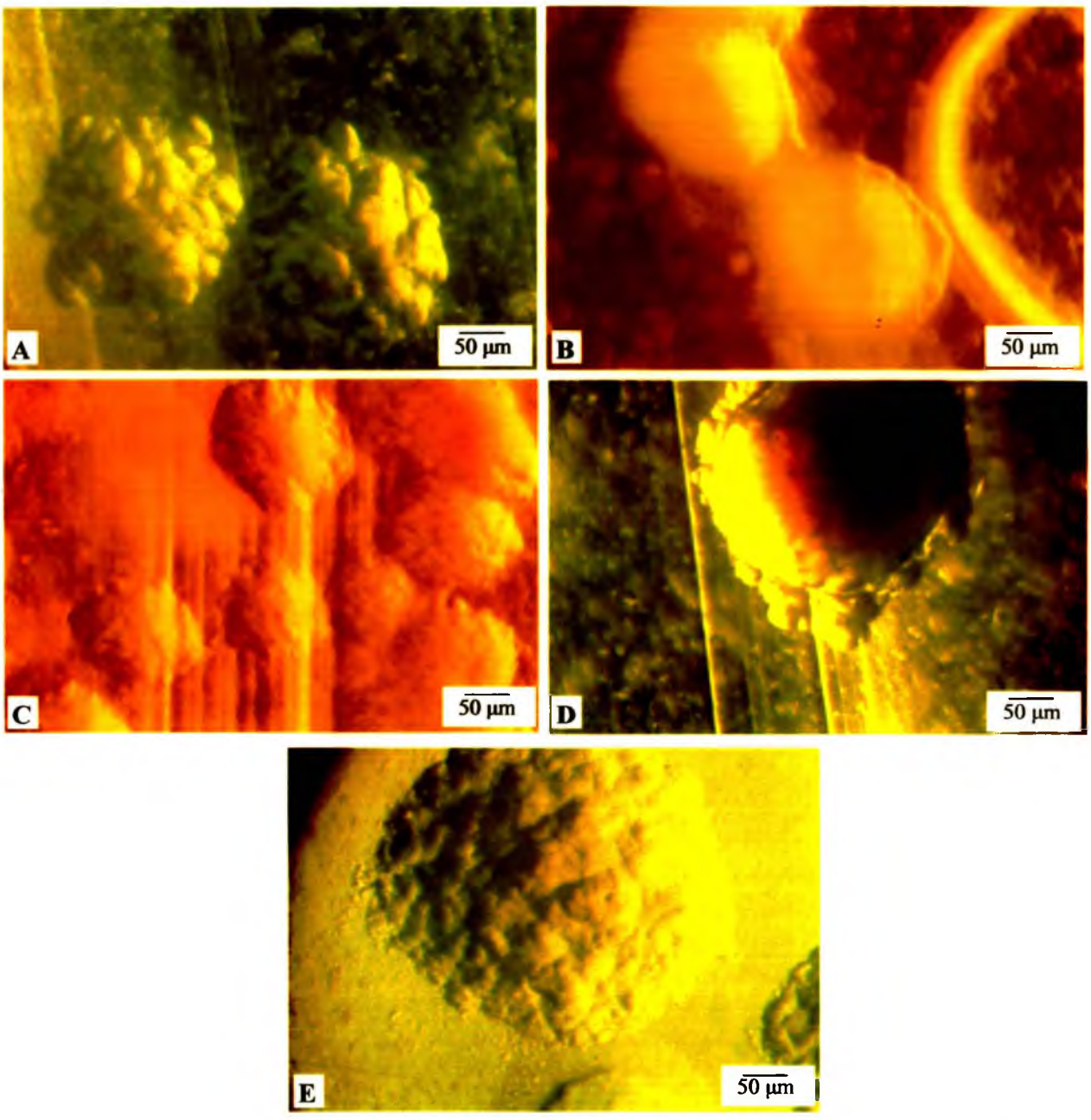

Fig .7. Colonies of the selected isolates as observed under microscope **A.** C2/2, **B.** GRK6, **C.** GRK7, **D.** C1/8 and **E.** C1/1. (Bar = 50µm).

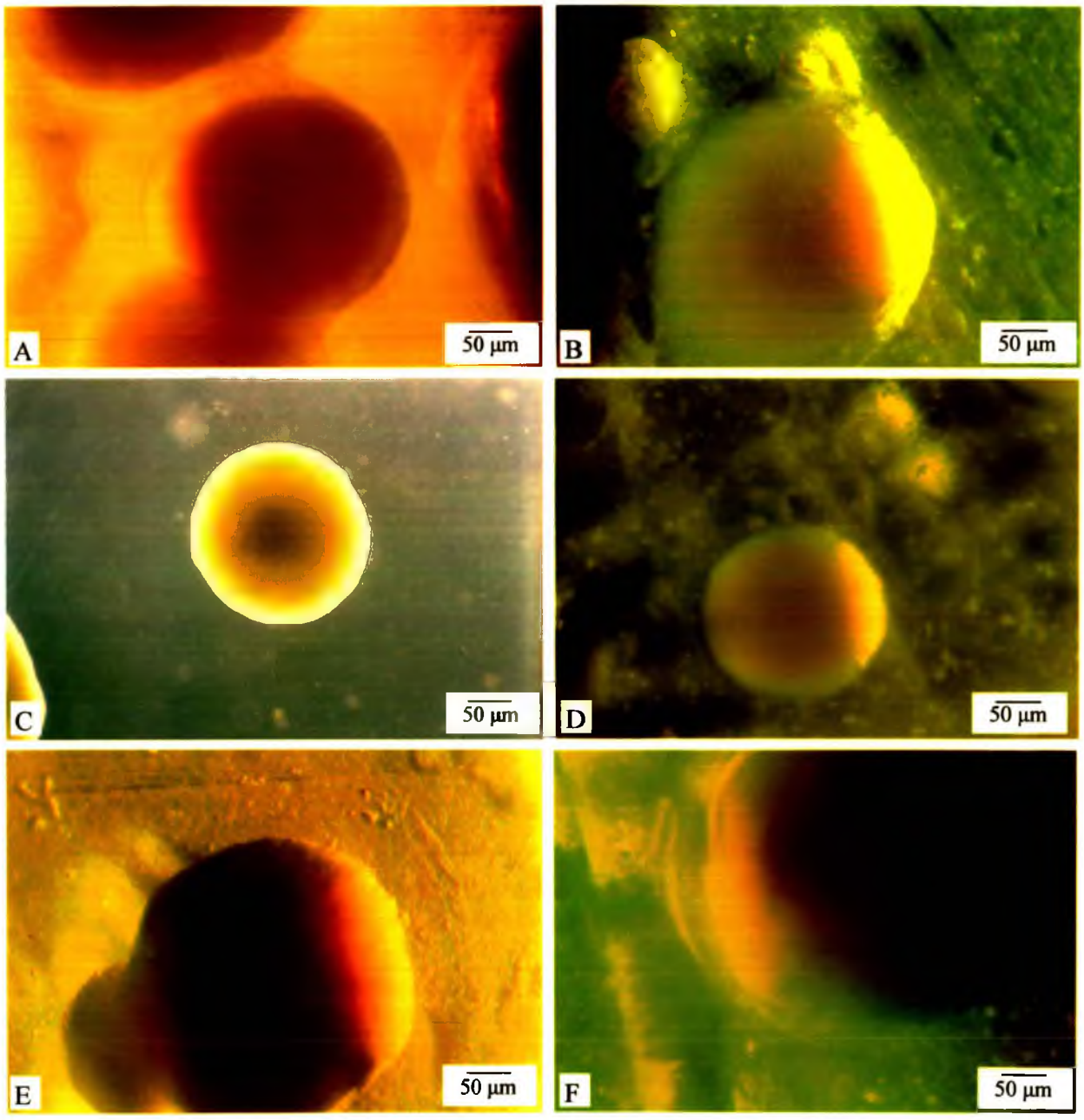

Fig 8. Colonies of the selected isolates as observed under microscope **A.** Cab 1, **B.** Cab2, **C.** Cab3, **D.** Cab5, **E.** Cab6 and **F.** Cab8 (Bar = 50µm).

Table 9: Microscopic observation of the selected aerobic isolates

AEROBIC ISOLATES			
Isolates	Vegetative cell	Isolates	Vegetative cell
C3/4	Rod shape, thick, short rod, arranged in single	DJJ3	Coccus form, arranged in chain and pair
C3/2	Rod shape, thick, long rod, arranged in single	FC2	Coccus form, arranged in chain and pair
C3/1b	Rod shape, thick, short, arranged in single	CAB1	Coccus form, arranged in tetrad and pair
C1/3	Rod shape, slender and long, arranged in chain, pair or single	CAB3	Rod shaped, thin to thick, arranged in single form
DJJ1	Coccus form, arranged in chain or pair	DJD4	Coccus form, arranged in chain and pair
DJJ4	Coccus form, arranged in chain or pair	TBY5	Coccus form, mostly arranged in pair and few chains present
DJD1	Rod shaped, slender , sometimes bent, arranged in pair or single		Coccus form, arranged in chain and pair

Table 10: Microscopic observation of the selected anaerobic isolates

ANAEROBIC ISOLATES			
Isolates	Vegetative cell	**Isolates**	Vegetative cell
C1/7a	Rod shaped, arranged in chain or pair	**C1/4**	Slender rod shaped, single, long rod
C1/5a	Rod shaped, arranged single	**GRK5**	Rod shaped, arranged in chain or pair
C1/7	Rod shaped, arranged in pair or single	**GRK6**	Rod shaped, arranged single or pair
C1/14	Rod shaped, arranged in cluster	**GRK7**	Rod shaped, arranged single or pair
C1/12	Slender rod shaped, arranged single	**C1/8**	Short rod, arranged in pair occasionally or single
C1/8(1)	Rod shaped, arranged in cluster	**C1/1**	Rod shaped, arranged in pair or single
C1/12a	Rod shaped, arranged in cluster	**C1/1b**	Rod shaped, arranged single
C1/12s	Short rod shaped, arranged in pair or single	**C2/6**	Rod shaped, arranged single
C1/10	Rod shaped, arranged in cluster or pair	**Cab1**	Rod shaped arranged single
C1/12b	Rod shaped, arranged single	**Cab2**	Short rod arranged in cluster
C1/16	Rod shaped, arranged in pair or single	**Cab3**	Long rod shaped, arranged single
DY(w)	Rod shaped, arranged single	**Cab4**	Long Rod shaped, arranged in cluster or single
DY(w)3	Rod shaped, arranged in cluster	**Cab5**	Rod shaped, arranged single
C1/2	Rod shaped, arranged single	**Cab6**	Rod shaped, arranged single
C2/2	Rod shaped, arranged single	**Cab8**	Rod shaped, arranged single

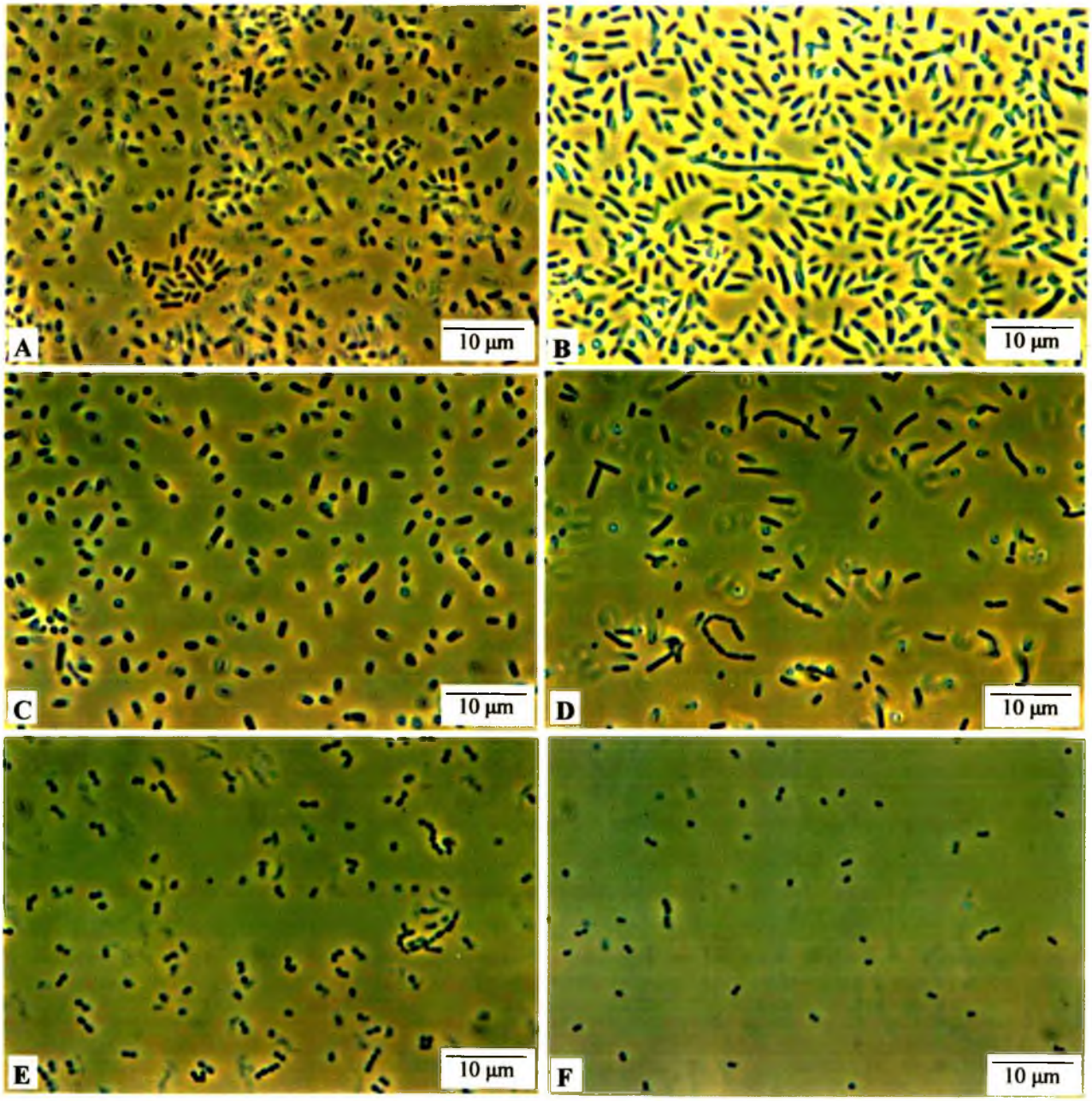

Fig 9. Vegetative cells of bacterial strains under phase contrast microscope. **A.** C3/4 **B.** C3/2 **C.** C3/1b **D.** C1/3 **E.** DJJ1and **F.** DJJ4 (Bar= 10µm).

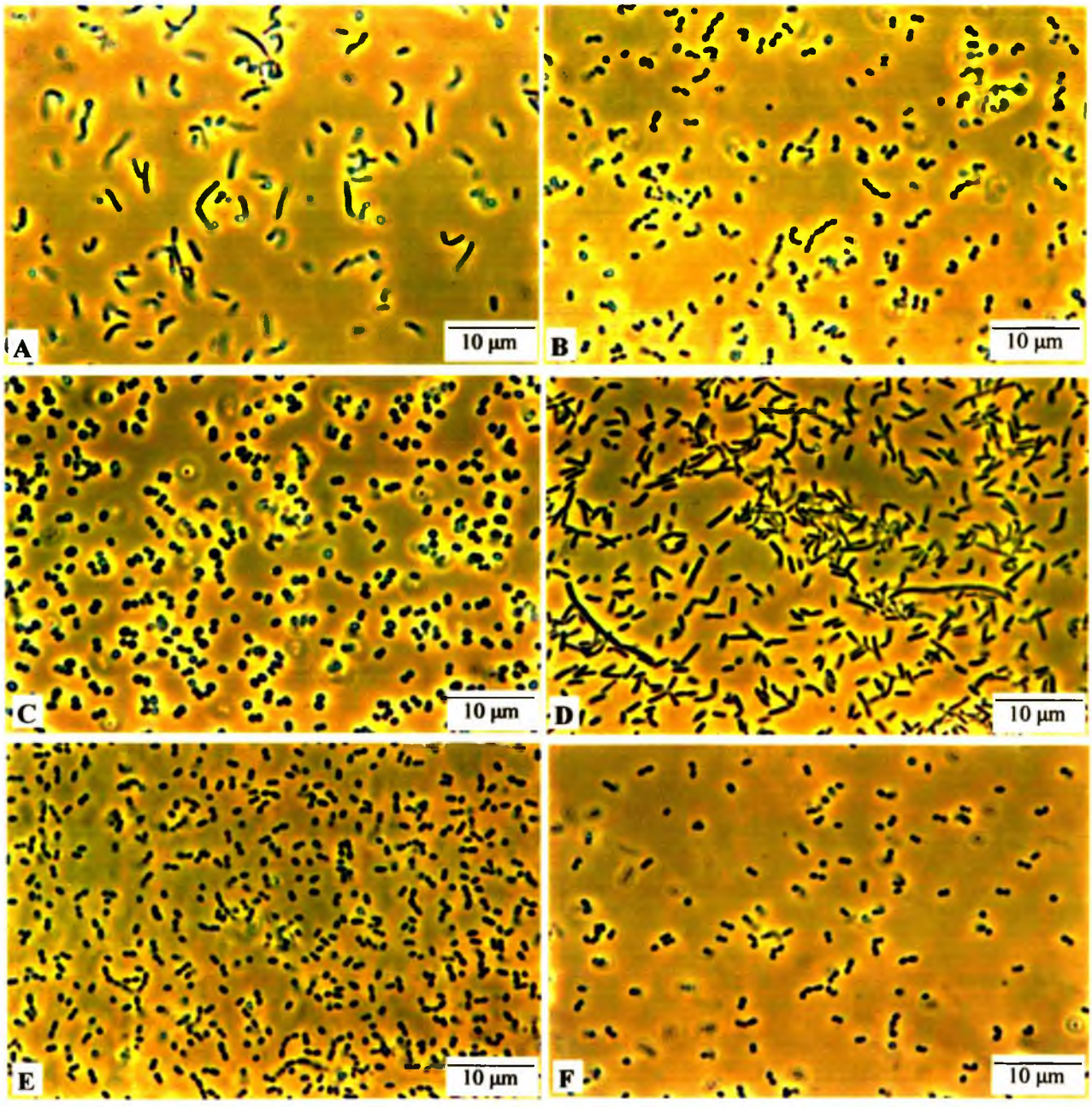

Fig.10. Vegetative cells of aerobic isolates under phase contrast microscope **A.** DJD1, **B.** DJJ3, **C.** CAB1, **D.** CAB3, **E.** DJD4 and **F.** TBY5 (Bar=10µm).

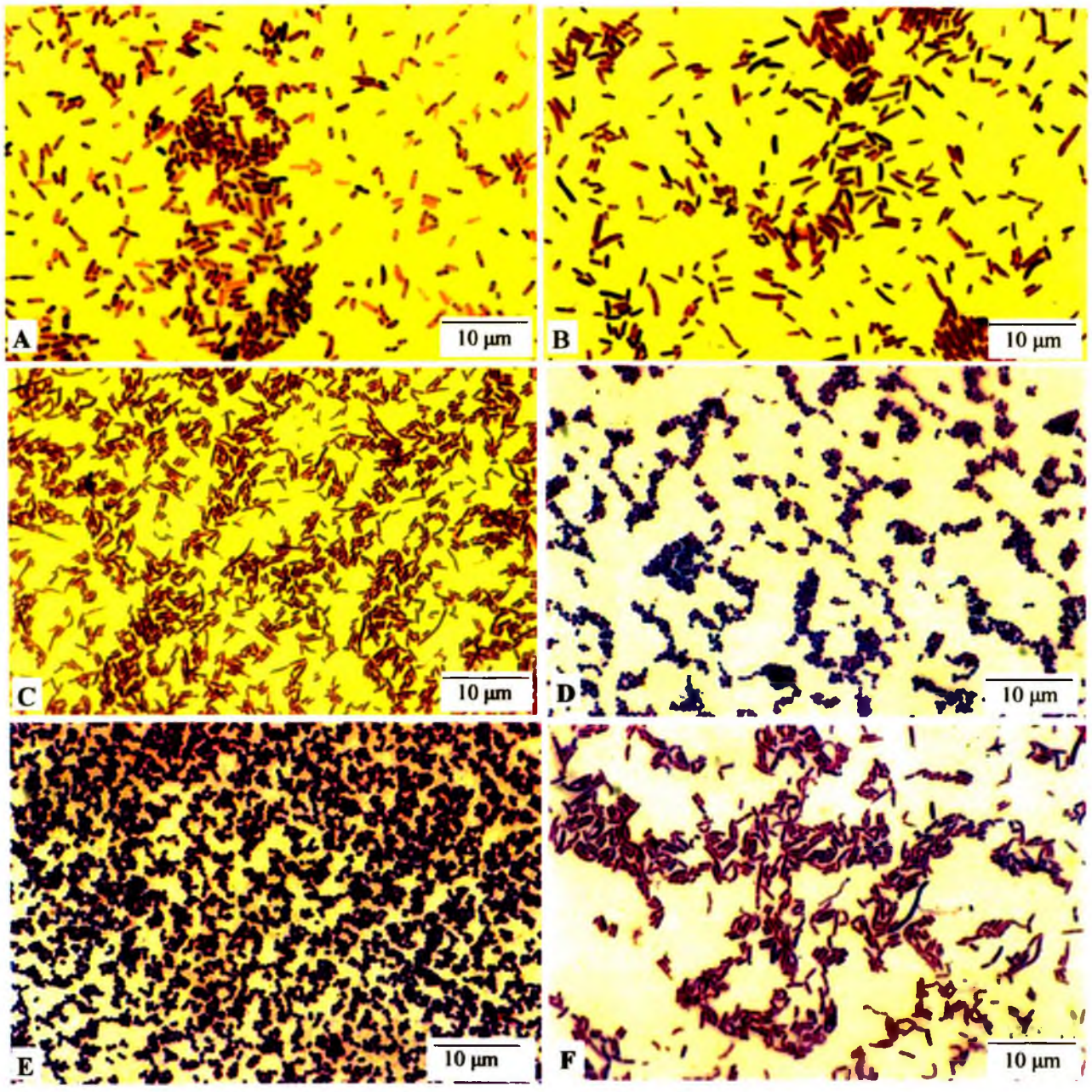

Fig .11. Photomicrographs showing gram staining reaction of selected aerobic isolates **A.** C3/4, **B.** C3/2, **C.** C1/3 **D.** DJJ1 **E.** DJJ4 and **F.** DJD1 (Bar= 10 µm).

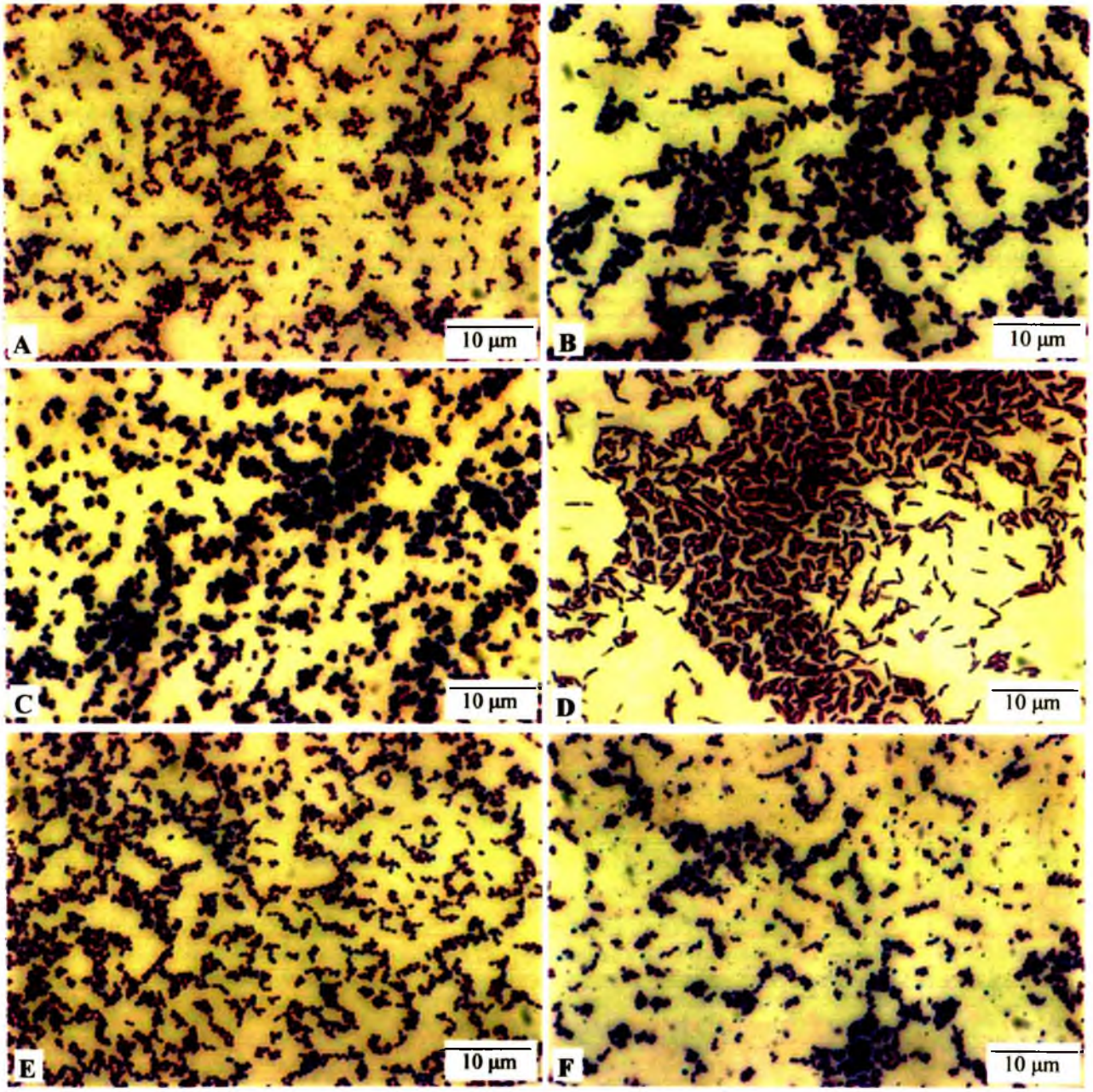

Fig 12. Photomicrographs showing Gram staining reaction of different bacterial strains **A.** DJJ3, **B.** FC2, **C.** CAB1, **D.** CAB3, **E.** DJD4 and **F.** TYB5 (Bar= 10µm).

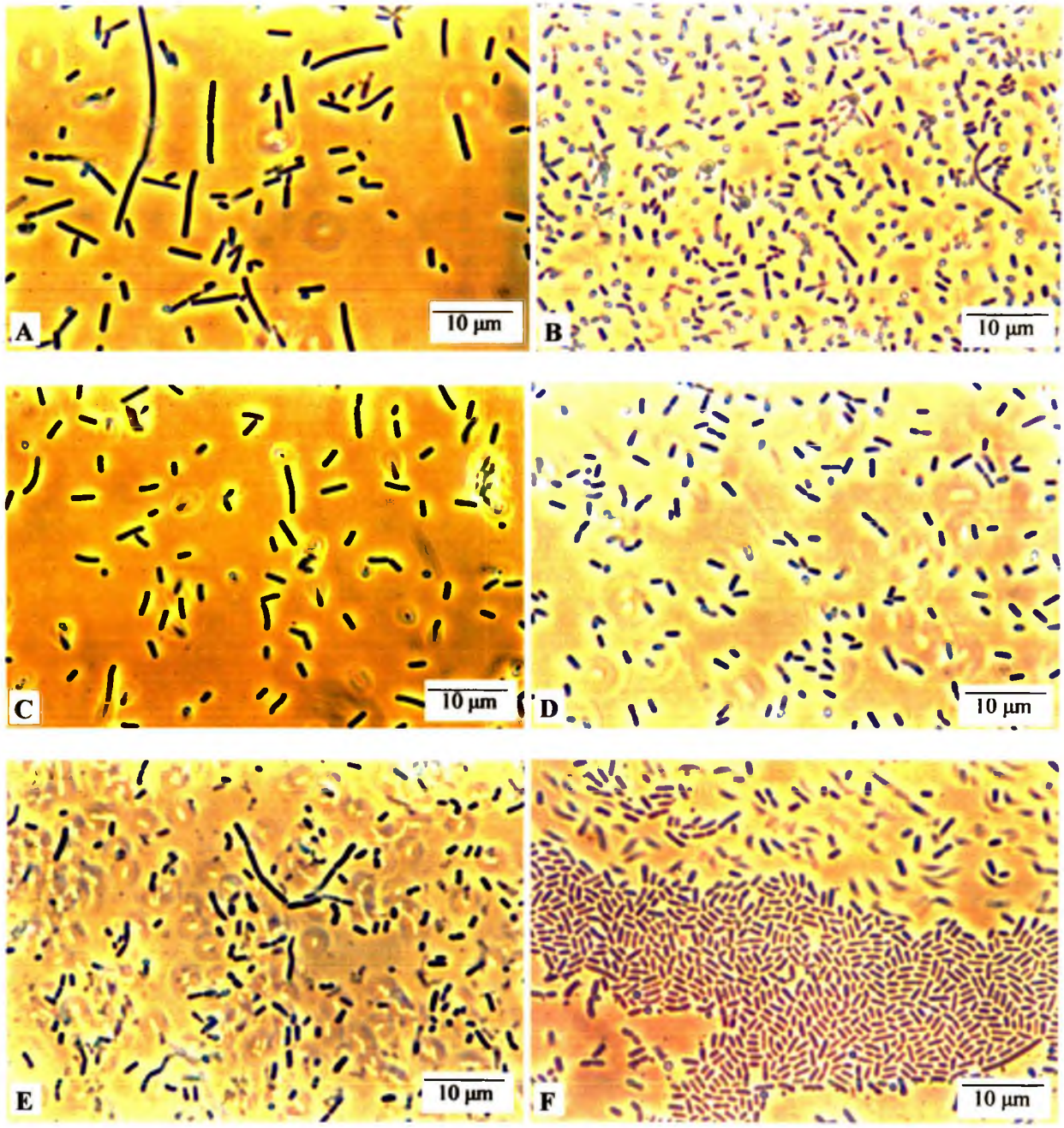

Fig. 13. Vegetative cells of aerobic isolates under phase contrast microscope **A.** C1/1, **B.** C1/4, **C.** C1/5a, **D.** C1/8(1), **E.** C1/8 and **F.** C1/10 (Bar = 10µm).

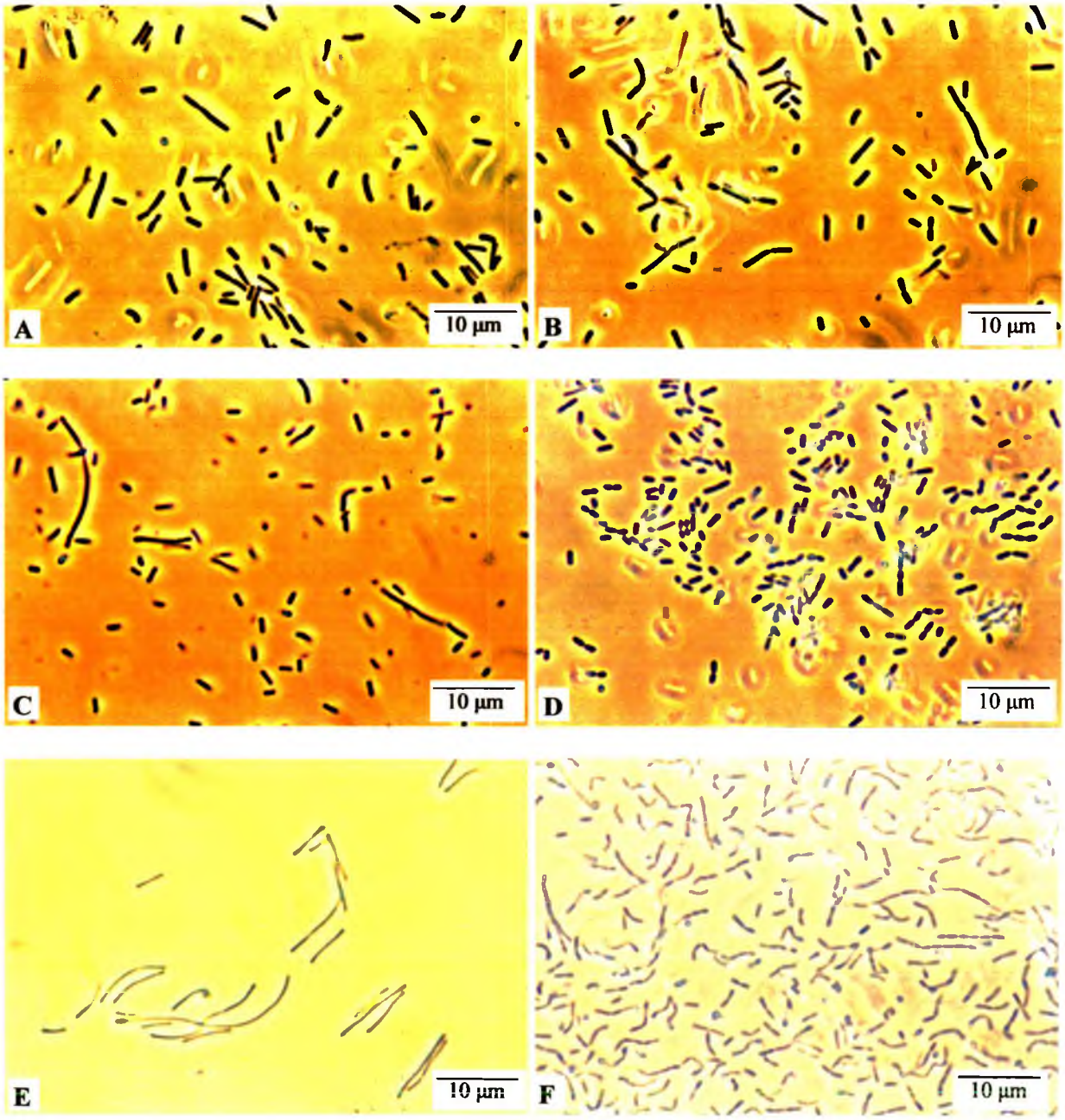

Fig. 14. Vegetative cells of aerobic isolates under phase contrast microscope **A.** C1/12, **B.** C1/12a, **C.** C1/12b, **D.** C1/12S, **E.** C1/14 and **F.** C1/16 (Bar = 10µm).

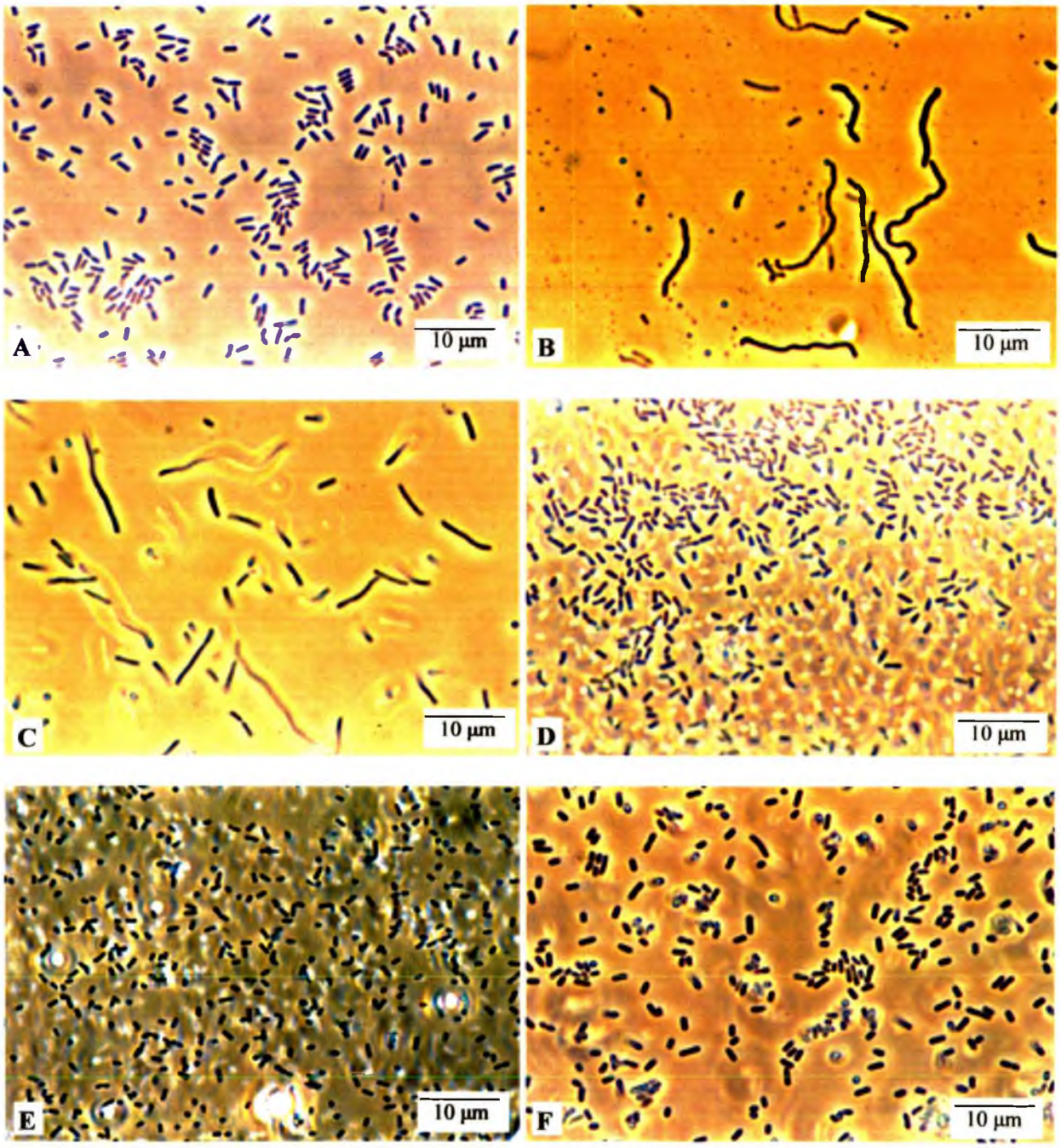

Fig. 15. Vegetative cells of aerobic isolates under phase contrast microscope **A.** Cab-1, **B.** Cab-3, **C.** Cab-4, **D**. Cab-6, **E.** DY (W) and **F.** DY (W)3 (Bar = 10µm).

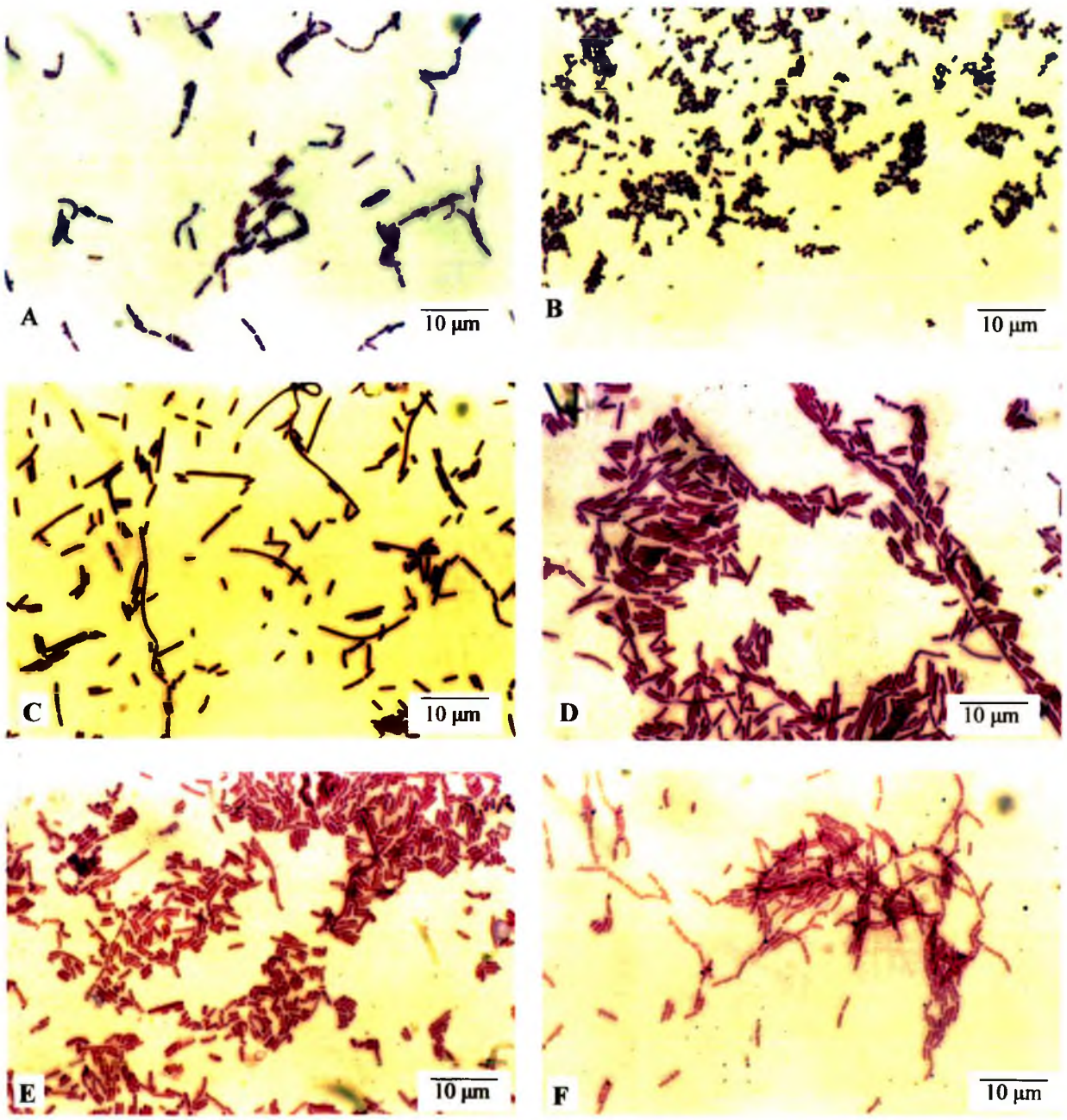

Fig. 16. Photomicrographs showing Gram staining reaction of isolate no. **A.** C1/1b, **B.** C1/2, **C.** C1/4, **D.** C1/5a, **E.** C1/7 and **F.** C1/7a (Bar = 10µm).

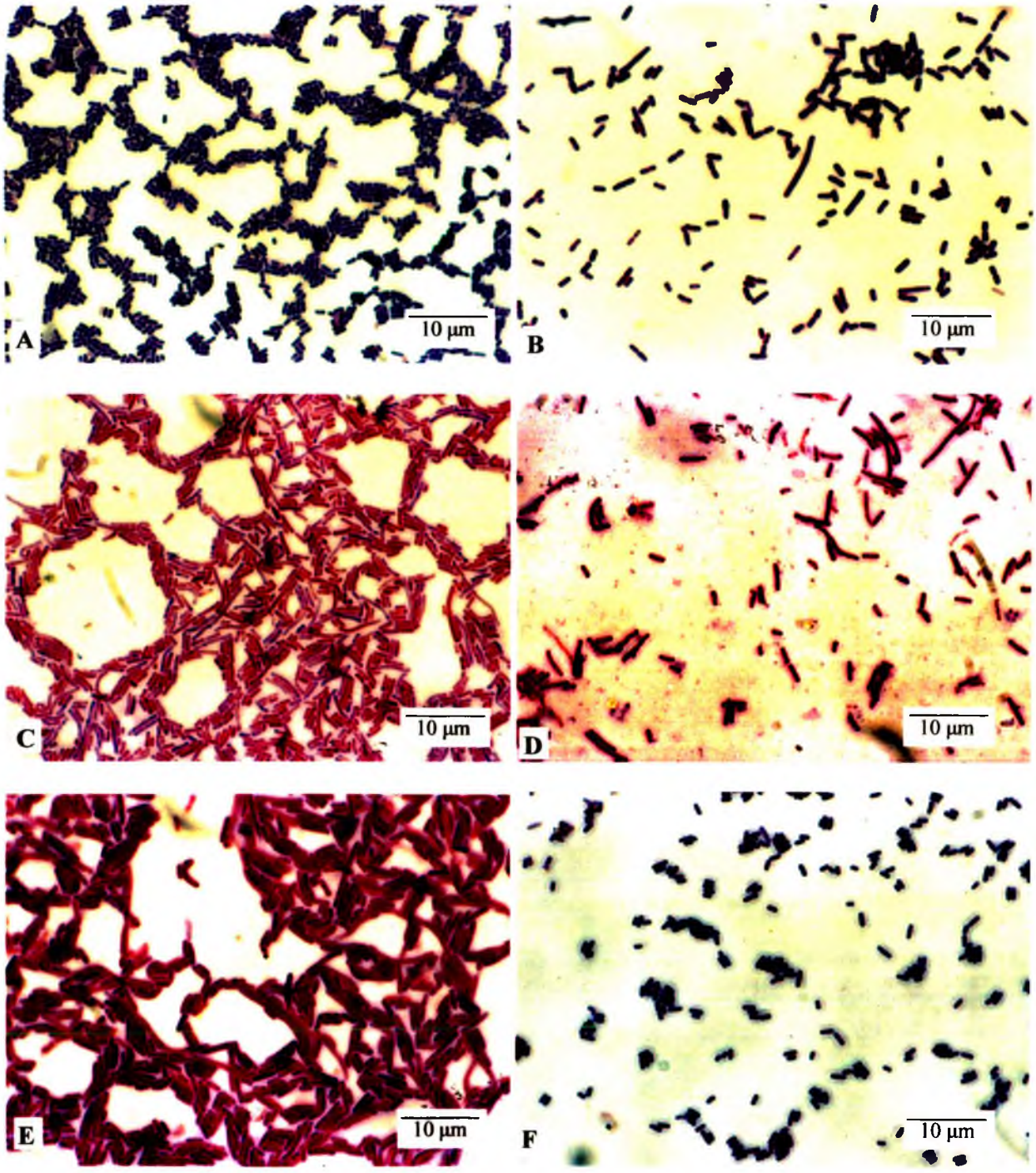

Fig 17. Gram staining reaction of isolate no. **A.** C1/8 (1), **B.** C1/8, **C.** C1/10, **D.** C1/12, **E.** C1/12a and **F.** C1/12b (Bar = 10µm).

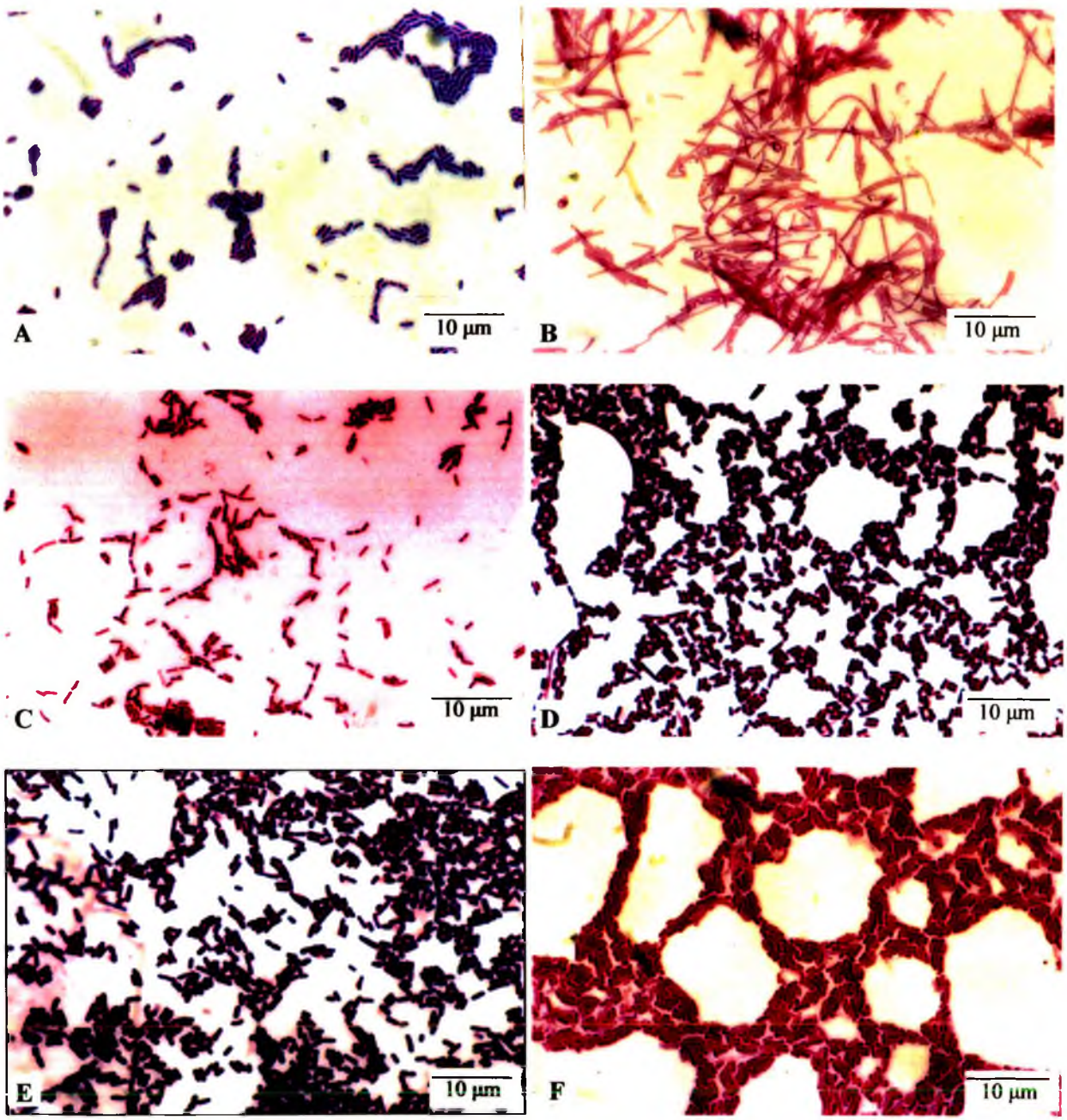

Fig 18. Photomicrographs showing Gram staining reaction of isolate no. **A**. C1/12S, **B.** C1/14, **C.** C1/16, **D.** C2/2, **E.** DY (W) and **F.** DY (W) 3 (Bar = 10µm).

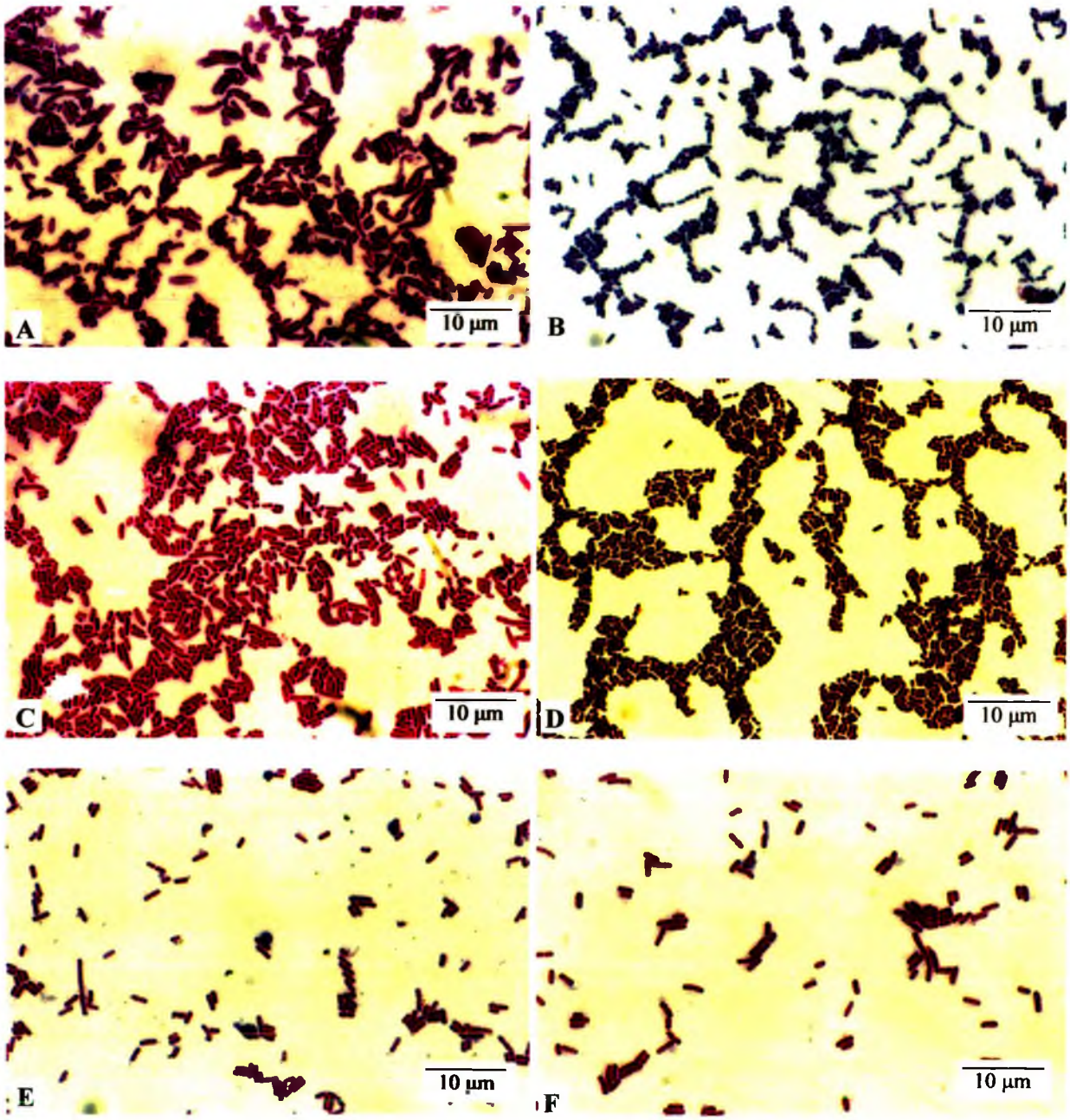

Fig 19. Photomicrographs showing Gram staining reaction of isolate no. **A**. Cab1, **B**. Cab4, **C.** Cab5, **D**. Cab8, **E.** GRK5 and **F.** GRK6 (Bar = 10µm).

Table 11: Different biochemical characteristics of aerobic isolates

Isolates	Catalase	Arginine hydrolysis	VP test	MR test	Protease test	Casein	Urease test	Nitrate reduction	Blood hemolysis test		Growth in presence of (%) bile salt		
									γ hemolytic	Non-hemolytic	0.5	1.0	2.0
C3/4	+	+	+	-	-	-	+	+	+	+	+	+	+
C3/2	+	+	+	-	-	-	+	+	-	+	+	+	+
C3/1b	+	+	+	-	-	+	+	+	+	+	+	+	+
C1/3	-	+	-	-	-	-	-	+	-	+	+	+	+
DJJ1	-	-	-	+	+	-	-	-	+	+	+	+	+
DJJ4	-	-	-	+	+	-	-	-	-	+	+	+	+
DJD1	-	-	-	+	+	-	-	-	+	+	+	+	+
DJJ3	-	-	-	+	+	+	-	-	+	+	+	+	GR
FC2	-	+	-	+	-	+	-	-	-	+	+	+	GR
CAB1	-	-	-	-	+	-	-	-	-	+	+	+	+
CAB3	-	-	-	-	+	-	-	-	-	+	+	+	+
DJD4	-	-	-	+	+	-	-	-	-	+	+	+	GR
TBY5	-	-	-	+	+	-	-	-	-	+	+	+	+

'+'positive, '-'Negative, 'GR' growth reduced

Table 12: Different biochemical characteristics of anaerobic isolates (cont.)

Isolates	Casein	MR test	Urease	Nitrate reduction	Arginine hydrolysis	Esculine	Protease	Blood agar hemolysis		Growth in presence of (%) bile salt		
								γ hemolytic	Non-hemolytic	0.5	1.0	2.0
C1/7a	-	+	-	+	+	-	+	+	+	+	+	+
C1/5a	+	+	+	-	-	-	+	+	+	+	+	+
C1/7	+	+	-	+	+	-	+	+	+	+	+	+
C1/14	+	-	-	-	-	-	+	-	+	+	+	+
C1/12	+	+	-	-	-	-	+	+	+	+	+	+
C1/8(1)	+	-	-	-	-	-	+	-	+	+	+	+
C1/12a	+	-	-	-	+	-	+	+	+	+	+	+
C1/12s	+	-	-	-	+	+	+	-	+	+	+	+
C1/10	-	-	-	-	-	+	+	+	+	+	+	+
C1/12b	+	+	-	-	-	-	+	-	+	+	+	+
C1/16	+	-	-	-	+	-	+	+	+	+	+	+
DY(w)	+	-	-	-	+	-	-	+	+	+	+	+
DY(w)3	+	-	-	-	-	+	-	+	+	+	+	+
C1/2	+	+	-	-	+	-	+	-	+	+	+	+
C2/2	+	+	-	-	+	-	-	-	+	+	+	GR

'+'positive, '-'Negative, 'GR' growth reduced

Table 12: Different biochemical characteristics of anaerobic isolates

Isolates	Casein	MR test	Urease	Nitrate reduction	Arginine hydrolysis	Esculine	Protease	Blood agar hemolysis		Growth in presence of (%) bile salt		
								γ hemolytic	Non-hemolytic	0.5	1.0	2.0
C1/4	+	-	-	-	+	+	-	-	+	+	+	GR
GRK5	+	-	-	-	-	+	-	-	+	+	+	GR
GRK6	+	-	-	-	+	+	-	-	+	+	+	GR
GRK7	+	-	-	-	-	+	-	-	+	+	+	+
C1/8	+	-	-	-	+	+	+	-	+	+	+	+
C1/1	+	-	-	+	-	+	+	-	+	+	+	+
C1/1b	+	-	-	-	-	-	+	-	+	+	+	+
C2/6	+	-	-	-	+	+	-	-	+	+	+	+
Cab1	-	-	-	+	-	+	+	+	+	+	+	+
Cab2	+	-	-	+	-	+	+	-	+	+	+	+
Cab3	+	-	-	-	-	-	+	-	+	+	+	+
Cab4	+	-	-	-	-	+	+	+	+	+	+	+
Cab5	+	-	-	+	+	+	-	-	+	+	+	+
Cab6	+	+	+	-	-	+	+	+	+	+	+	+
Cab8	+	+	-	-	+	+	+	+	+	+	+	+

'+'positive, '-'Negative, 'GR' growth reduced

Table 13: Production of acid from different carbohydrate sources by aerobic isolates (cont.):

Isolates	Xylose	Glucose (dextrose)	fructose (levulose)	Galactose	Mannose	Rhamnose	Sorbose	Raffinose
C3/4	-	-	-	-	-	+	+	-
C3/2	-	-	-	-	-	+	-	-
C3/1b	-	-	-	-	-	+	+	-
C1/3	-	+	+	-	-	+	-	-
DJJ1	+	+	+	+	+	-	-	-
DJJ4	+	+	+	+	+	-	-	+
DJD1	-	+	+	+	+	-	-	+
DJJ3	+	+	+	+	+	-	-	-
FC2	-	+	+	-	+	-	-	-
CAB1	+	+	+	+	+	-	-	-
CAB3	-	+	+	+	+	-	-	-
DJD4	-	-	+	+	+	-	-	-
TBY5	-	-	+	+	+	-	-	-

'+'positive, '-'Negative

Table 13: Production of acid from different carbohydrate sources by aerobic isolates (cont.):

Isolates	Sorbitol	glycerol	Inositol	Mannitol	Dulcitol	Adonitol	Starch	Inulin
C3/4	-	+	-	-	+	-	-	-
C3/2	-	+	-	-	-	-	-	-
C3/1b	-	+	-	-	+	-	-	-
C1/3	+	-	-	+	-	-	-	+
DJJ1	-	-	-	+	-	-	-	+
DJJ4	-	-	-	+	-	-	-	+
DJD1	+	-	-	+	-	-	-	+
DJJ3	-	-	-	+	-	-	-	+
FC2	-	-	-	+	-	-	-	+
CAB1	-	-	-	-	-	-	-	+
CAB3	+	-	-	+	-	-	-	+
DJD4	-	-	-	+	-	-	-	+
TBY5	-	-	-	-	-	-	-	+

'+'positive, '-'Negative

Table 13: Production of acid from different carbohydrate sources by aerobic isolates:

Isolates	Sucrose	Maltose	Lactose	Cellobiose	Trehalose	Melibiose	Salicin	Glutamate
C3/4	-	+	+	+	-	-	-	-
C3/2	-	+	+	+	-	-	-	-
C3/1b	-	+	+	+	-	-	-	-
C1/3	+	+	+	+	+	-	-	-
DJJ1	+	+	+	+	+	-	+	-
DJJ4	+	+	+	+	-	-	-	-
DJD1	+	+	+	+	+	-	-	-
DJJ3	+	+	+	+	-	-	-	-
FC2	+	+	+	+	-	-	-	-
CAB1	+	+	+	+	+	-	-	-
CAB3	+	-	+	+	+	-	-	-
DJD4	+	-	+	+	-	-	-	-
TBY5	+	+	+	-	+	-	+	-

'+'positive, '-'Negative

Table 14: Production of acid from different carbohydrate sources by anaerobic isolates (cont.):

Isolates	Xylose	Glucose (dextros)	fructose (levulos)	Galactose	Mannose	Rhamnose	Sorbose	Raffinose
C1/7a	-	+	+	+	+	+	+	+
C1/5a	+	+	+	+	-	-	-	-
C1/7	+	+	+	+	-	-	+	-
C1/14	-	+	+	+	+	+	+	+
C1/12	+	+	+	+	-	-	-	-
C1/8(1)	+	+	+	+	-	-	-	-
C1/12a	+	+	+	+	-	+	-	-
C1/12s	+	+	+	+	+	-	+	-
C1/10	+	+	+	+	-	-	+	-
C1/12b	+	+	+	+	-	-	+	-
C1/16	-	+	+	+	+	+	+	+
DY(w)	-	+	+	+	-	+	-	-
DY(w)3	+	+	+	+	-	-	-	-
C1/2	-	+	+	+	-	+	-	-
C2/2	-	+	+	+	-	+	-	-

'+'positive, '-'Negative

Table 14: Production of acid from different carbohydrate sources by anaerobic isolates (cont.):

Isolates	Xylose	Glucose(dextrose)	fructose (levulose)	Galactose	Mannose	Rhamnose	Sorbose	Raffinose
C1/4	-	+	+	+	-	-	+	-
GRK5	+	+	+	+	-	-	+	+
GRK6	+	+	+	+	+	-	-	-
GRK7	+	+	+	+	-	-	+	-
C1/8	-	+	+	+	-	-	-	-
C1/1	+	+	+	+	-	-	+	+
C1/1b	-	+	+	+	+	+	-	+
C2/6	-	+	+	+	-	-	+	+
Cab1	+	+	+	+	+	+	-	+
Cab2	-	+	+	+	-	-	-	-
Cab3	-	+	+	+	-	-	-	-
Cab4	-	+	+	+	-	-	-	-
Cab5	+	+	+	-	+	-	-	+
Cab6	-	+	+	+	-	-	-	-
Cab8	+	+	+	+	-	-	-	-

'-' negative, '+' positive

Table 14: Production of acid from different carbohydrate sources by anaerobic isolates (Cont.):

Isolates	Sorbitol	Glycerol	Inositol	Mannitol	Dulcitol	Adonitol	Starch	Inulin
C1/7a	+	+	+	+	-	-	+	+
C1/5a	-	-	+	+	-	-	-	+
C1/7	-	-	+	+	+	+	-	+
C1/14	+	+	+	+	-	+	+	+
C1/12	-	-	+	+	+	+	-	+
C1/8(1)	-	-	-	+	+	+	-	+
C1/12a	+	-	-	+	+	+	+	+
C1/12s	-	-	+	+	+	+	+	+
C1/10	-	-	+	+	+	+	-	+
C1/12b	-	-	+	+	+	+	-	+
C1/16	+	+	+	+	-	+	+	+
DY(w)	+	-	-	+	-	+	+	+
DY(w)3	-	-	+	+	+	-	-	+
C1/2	+	-	-	+	-	-	+	+
C2/2	-	-	-	-	-	-	+	+

'-' negative, '+' positive

Table 14: Production of acid from different carbohydrate sources by anaerobic isolates (Cont.):

Isolates	Sorbitol	Glycerol	Inositol	Mannitol	Dulcitol	Adonitol	Starch	Inulin
C1/4	-	-	+	+	+	+	-	+
GRK5	+	+	+	-	+	+	-	+
GRK6	-	-	-	-	-	+	-	+
GRK7	-	-	+	+	+	+	-	+
C1/8	+	-	-	+	-	-	+	+
C1/1	+	-	+	+	+	+	-	+
C1/1b	+	+	-	+	-	+	+	+
C2/6	-	-	-	+	-	-	+	+
Cab1	+	+	+	+	-	-	+	+
Cab2	-	-	+	+	-	-	-	+
Cab3	-	-	-	+	+	-	-	-
Cab4	-	-	-	+	+	-	-	+
Cab5	+	-	-	-	-	-	-	+
Cab6	-	-	-	+	+	-	-	+
Cab8	-	-	-	+	+	-	-	+

'-' negative, '+' positive

Table 14: Production of acid from different carbohydrate sources by anaerobic isolates (Cont.):

Isolates	Sucrose	Maltose	Lactose	Cellobiose	Trehalose	Melibiose	Salicin	Glutamate
C1/7a	+	-	+	+	+	+	+	+
C1/5a	+	+	+	-	-	-	-	-
C1/7	+	+	+	-	-	-	-	-
C1/14	+	-	+	+	+	+	+	+
C1/12	+	+	+	-	-	-	-	-
C1/8(1)	+	+	+	-	-	-	-	-
C1/12a	-	+	+	+	+	-	-	-
C1/12s	+	+	+	-	-	+	+	-
C1/10	+	+	+	-	-	-	-	-
C1/12b	+	+	+	-	-	-	-	-
C1/16	+	+	+	+	+	+	+	+
DY(w)	+	+	+	-	-	+	-	-
DY(w) 3	+	+	+	-	-	-	-	-
C1/2	+	+	+	+	+	+	+	+
C2/2	+	+	+	+	-	+	-	-

'-' negative, '+' positive

Table 14: Production of acid from different carbohydrate sources by anaerobic isolates (Cont.):

Isolates	Sucrose	Maltose	Lactose	Cellobiose	Trehalose	Melibiose	Salicin	Glutamate
C1/4	+	+	+	-	-	-	-	-
GRK5	+	+	+.	+	-	+	-	-
GRK6	+	+	+	-	-	-	-	-
GRK7	+	+	+	-	-	-	-	-
C1/8	+	+	+	+	+	+	-	+
C1/1	+	+	+	-	-	+	+	-
C1/1b	+	-	+	+	+	+	+	+
C2/6	+	+	+	-	-	+	-	-
Cab1	+	+	+	+	+	+	+	+
Cab2	+	+	+	-	-	-	-	-
Cab3	+	+	-	-	-	-	-	-
Cab4	+	+	+	-	-	-	-	-
Cab5	-	-	-	-	-	+	+	-
Cab6	+	+	+	-	-	-	-	-
Cab8	+	+	+	-	-	-	-	-

'-' negative, '+' positive

Table 15: Test with Litmus milk (cont.):

Isolates	Acid production	Acid clot	Gas	Sweet clot	Casein	Citrate
C3/4	+	+	+	-	+	-
C3/2	+	+	+	-	+	-
C3/1b	+	+	+	-	+	-
C1/3	+	+	-	-	+	-
DJJ1	+	-	-	-	-	-
DJJ4	+	-	-	-	-	-
DJD1	+	+	-	-	-	-
DJJ3	+	-	-	-	-	-
FC2	+	+	-	+	-	-
CAB1	+	-	-	-	-	-
CAB3	+	+	+	-	+	-
DJD4	-	-	-	-	-	-
TBY5	+	-	-	-	-	-

'-' negative, '+' positive

Table 15: Test with Litmus milk (cont.):

Isolates	Acid production	Acid clot	Gas	Sweet clot	Casein	Citrate
C1/7a	+	+	-	-	-	-
C1/5a	+	+	-	-	+	-
C1/7	+	+	-	-	+	-
C1/14	+	+	-	-	+	-
C1/12	+	+	-	-	+	-
C1/8(1)	+	+	-	-	+	-
C1/12a	+	+	-	-	+	-
C1/12s	+	+	+	-	+	-
C1/10	-	-	-	-	-	-
C1/12b	+	+	+	-	+	-
C1/16	+	+	-	-	+	-
DY(w)	+	+	-	-	+	-
DY(w)3	+	+	-	-	+	-
C1/2	+	+	-	-	+	-
C2/2	+	+	-	-	+	-

'-' negative, '+' positive

Table 15: Test with Litmus milk:

Isolates	Acid production	Acid clot	Gas	Sweet clot	Casein	Citrate
C1/4	+	+	+	-	+	-
GRK5	+	+	+	-	+	-
GRK6	+	+	+	-	+	-
GRK7	+	+	+	-	+	-
C1/8	+	+	+	-	+	-
C1/1	+	+	-	-	+	-
C1/1b	+	+	+	-	+	-
C2/6	+	+	-	-	+	-
Cab1	+	-	-	-	-	-
Cab2	+	+	+	-	+	-
Cab3	+	+	+	-	+	-
Cab4	+	+	+	-	+	-
Cab5	+	+	+	-	+	-
Cab6	+	+	+	-	+	-
Cab8	+	+	+	-	+	-

'-' negative, '+' positive

Table 16: Oxygen requirement test of selected isolates (Cont.):

Isolates	Type of organisms in relation to oxygen
C3/4	Facultative anaerobe – produced gas from glucose
C3/2	Facultative anaerobe – produced gas from glucose
C3/1b	Facultative anaerobe – produced gas from glucose
C1/3	Facultative anaerobe
DJJ1	Facultative anaerobe
DJJ4	Facultative anaerobe
DJD1	Facultative anaerobe
DJJ3	Obligate aerobe
FC2	Aerotolerant anaerobe
CAB1	Aerotolerant anaerobe
CAB3	Facultative anaerobe
DJD4	Obligate aerobe
TBY5	Microaerophilic

Table 16: Oxygen requirement test of selected isolates:

Isolates	Type of organisms in relation to oxygen	Isolates	Type of organisms in relation to oxygen
C1/7a	Aerotolerant anaerobe	C1/4	Aerotolerant anaerobe Gas produced from glucose
C1/5a	Aerotolerant anaerobe	GRK5	Aerotolerant anaerobe Gas produced from glucose
C1/7	Aerotolerant anaerobe	GRK6	Aerotolerant anaerobe Gas produced from glucose
C1/14	Microaerophilic	GRK7	Aerotolerant anaerobe
C1/12	Aerotolerant anaerobe	C1/8	Aerotolerant anaerobe
C1/8(1)	Aerotolerant anaerobe	C1/1	Aerotolerant anaerobe
C1/12a	Aerotolerant anaerobe	C1/1b	Aerotolerant anaerobe
C1/12s	Aerotolerant anaerobe	C2/6	Aerotolerant anaerobe
C1/10	Aerotolerant anaerobe	Cab1	Aerotolerant anaerobe
C1/12b	Aerotolerant anaerobe	Cab2	Aerotolerant anaerobe
C1/16	Aerotolerant anaerobe	Cab3	Microaerophilic
DY(w)	Aerotolerant anaerobe Gas produced from glucose	Cab4	Aerotolerant anaerobe
DY(w)3	Aerotolerant anaerobe	Cab5	Aerotolerant anaerobe Gas produced from glucose
C1/2	Aerotolerant anaerobe Gas produced from glucose	Cab6	Aerotolerant anaerobe
C2/2	Aerotolerant anaerobe	Cab8	Aerotolerant anaerobe

Table 17: Growth response of anaerobic bacteria at different temperature (Cont.)

	TEMPERATURE (^{O}C)						
Isolates	5°	10°	15°	25°	37°	45°	50°
C1/7a	+	+	+	+	+	+	+
C1/5a	+	+	+	+	+	+	-
C1/7	+	+	+	+	+	+	+
C1/14	+	+	+	+	+	+	-
C1/12	+	+	+	+	+	+	+
C1/8(1)	+	+	+	+	+	+	+
C1/12a	+	+	+	+	+	+	+
C1/12s	-	+	+	+	+	+	-
C1/10	+	+	+	+	+	+	+
C1/12b	+	+	+	+	+	+	+
C1/16	+	+	+	+	+	+	+
DY(w)	+	+	+	+	+	+	+
DY(w)3	+	+	+	+	+	+	+
C1/2	+	+	+	+	+	+	+
C2/2	+	+	+	+	+	+	+

'-' no growth, '+' growth present

Table 17: Growth response of anaerobic bacteria at different temperature

	TEMPERATURE (^{O}C)						
Isolates	5°	10°	15°	25°	37°	45°	50°
C1/4	+	+	+	+	+	+	-
GRK5	+	+	+	+	+	+	-
GRK6	+	+	+	+	+	+	+
GRK7	+	+	+	+	+	+	+
C1/8	+	+	+	+	+	+	+
C1/1	+	+	+	+	+	+	+
C1/1b	-	+	+	+	+	+	-
C2/6	+	+	+	+	+	+	+
Cab1	+	+	+	+	+	+	-
Cab2	+	+	+	+	+	+	-
Cab3	+	+	+	+	+	+	+
Cab4	+	+	+	+	+	+	+
Cab5	+	+	+	+	+	+	+
Cab6	+	+	+	+	+	-	-
Cab8	+	+	+	+	+	+	-

'-' no growth, '+' growth present

Table 18: Growth response of aerobic bacteria at different temperature

	TEMPERATURE (^{0}C)						
C1/7a	5°	10°	25°	30°	37°	45°	50°
C1/5a	+	+	+	+	+	+	+
C1/7	+	+	+	+	+	+	+
C1/14	+	+	+	+	+	+	+
C1/12	+	+	+	+	+	+	+
C1/8(1)	+	+	+	+	+	+	+
C1/12a	+	+	+	+	+	+	+
C1/12s	+	+	+	+	+	+	+
C1/10	+	+	+	+	+	+	+
C1/12b	+	+	+	+	+	+	+
C1/16	+	+	+	+	+	+	+
DY(w)	+	+	+	+	+	+	+
DY(w)3	+	+	+	+	+	-	-
C1/2	+	+	+	+	+	+	+

'-' no growth, '+' growth present

Table 19: Growth response of isolated aerobic bacteria at different pH

	pH						
Isolates	4.0	4.5	5.5	6.5	7.5	8.5	9.5
C3/4	+	+	+	+	+	+	+
C3/2	+	+	+	+	+	+	+
C3/1b	+	+	+	+	+	+	+
C1/3	+	+	+	+	+	+	+
DJJ1	+	+	+	+	+	+	+
DJJ4	+	+	+	+	+	+	+
DJD1	+	+	+	+	+	+	+
DJJ3	+	+	+	+	+	+	+
FC2	+	+	+	+	+	+	+
CAB1	+	+	+	+	+	+	+
CAB3	+	+	+	+	+	+	+
DJD4	+	+	+	+	+	+	+

'-' no growth, '+' growth present

Table 20: Growth response of isolated anaerobic bacteria at different pH (cont.)

	pH				
Isolates	3.5	4.5	5.5	6.5	7.5
C1/7a	+	+	+	+	+
C1/5a	+	+	+	+	+
C1/7	+	+	+	+	+
C1/14	+	+	+	+	+
C1/12	+	+	+	+	+
C1/8(1)	+	+	+	+	+
C1/12a	+	+	+	+	+
C1/12s	+	+	+	+	+
C1/10	+	+	+	+	+
C1/12b	+	+	+	+	+
C1/16	+	+	+	+	+
DY(w)	+	+	+	+	+
DY(w)3	+	+	+	+	+
C1/2	+	+	+	+	+
C2/2	+	+	+	+	+

'+' growth present

Table 20: Growth response of isolated anaerobic bacteria at different pH:

	pH				
Isolates	3.5	4.5	5.5	6.5	7.5
C1/4	+	+	+	+	+
GRK5	+	+	+	+	+
GRK6	+	+	+	+	+
GRK7	+	+	+	+	+
C1/8	+	+	+	+	+
C1/1	+	+	+	+	+
C1/1b	+	+	+	+	+
C2/6	+	+	+	+	+
Cab1	+	+	+	+	+
Cab2	+	+	+	+	+
Cab3	+	+	+	+	+
Cab4	+	+	+	+	+
Cab5	+	+	+	+	+
Cab6	+	+	+	+	+
Cab8	+	+	+	+	+

'+' growth present

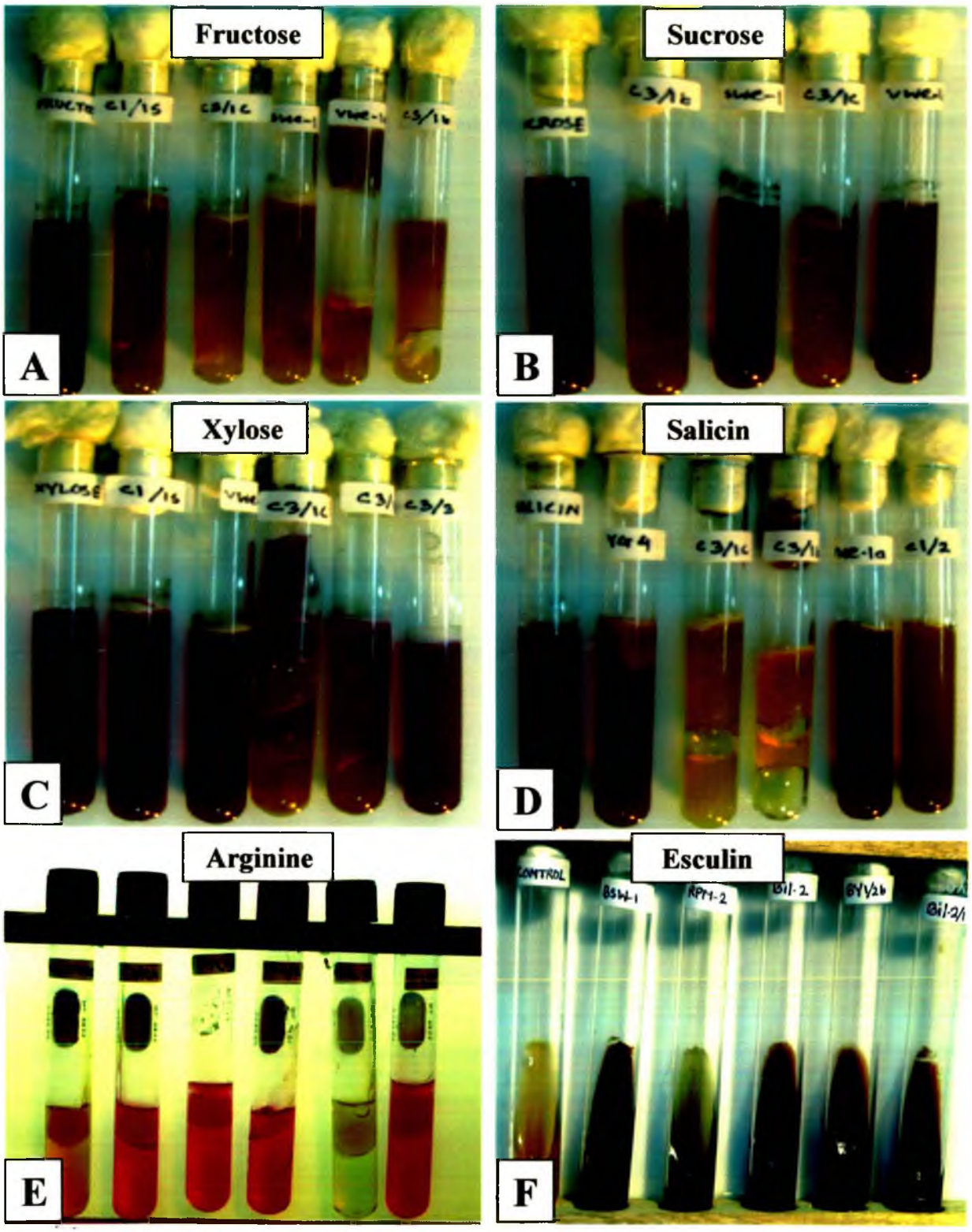

Fig. 20. Fermentation test results of the selected isolates (A-D) and results of the arginine and esculin hydrolysis test (E-F).

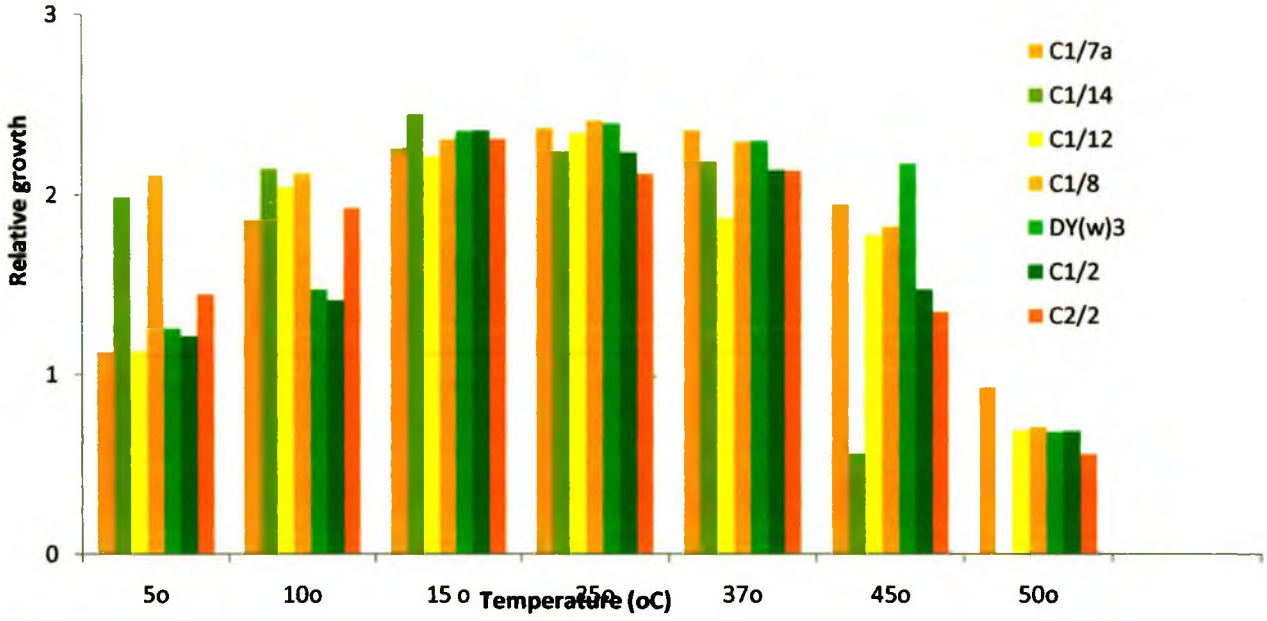

Fig 21 Growth response of anaerobic bacteria- C1/7a, C1/14, C1/12, C1/8, DY(w)3, C1/2 and C2/2 at different temperature (°C).

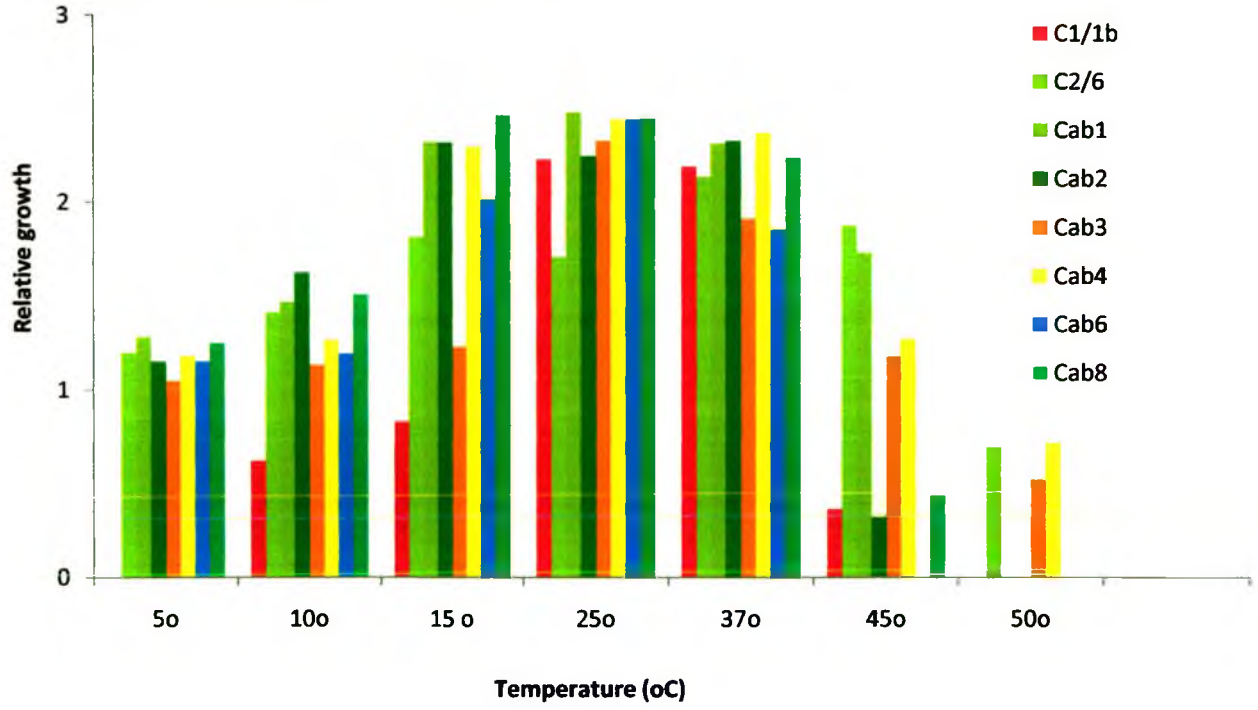

Fig 22 Growth response of anaerobic bacteria- C1/1b, C2/6, Cab1, Cab2, Cab3, Cab4, Cab6 and Cab8 at different temperature (°C).

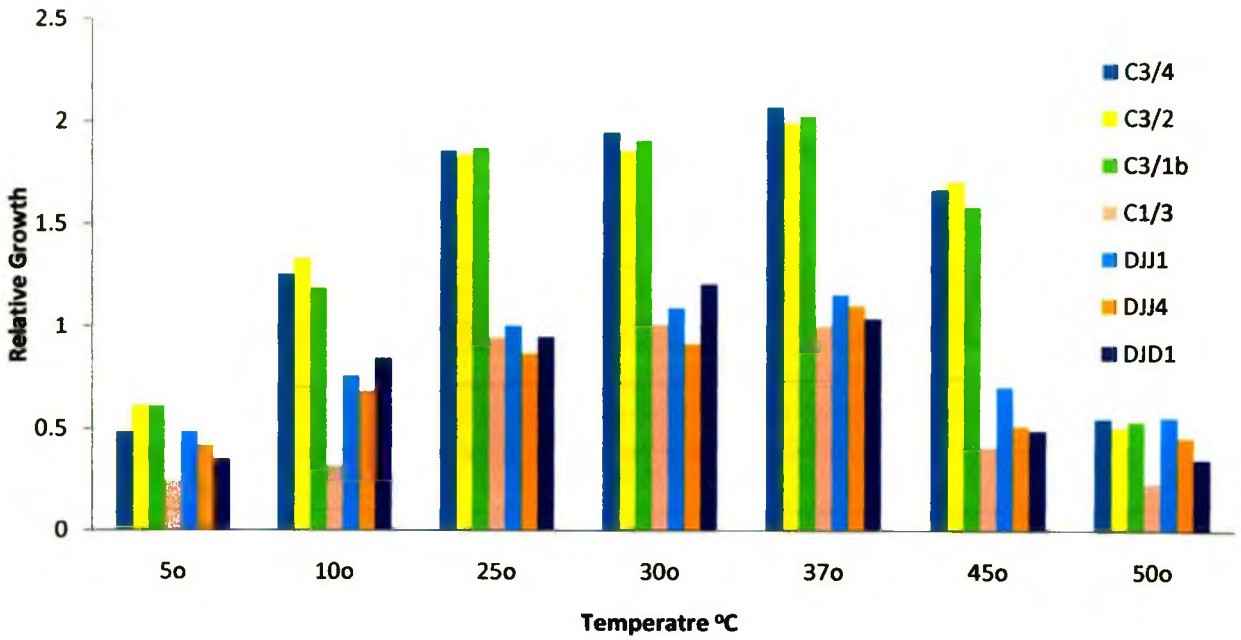

Fig 23 Growth response of aerobic isolates (C3/4, C3/2, C3/1b, C1/3, DJJ1, DJJ4 and DJD1) at different temperature (°C).

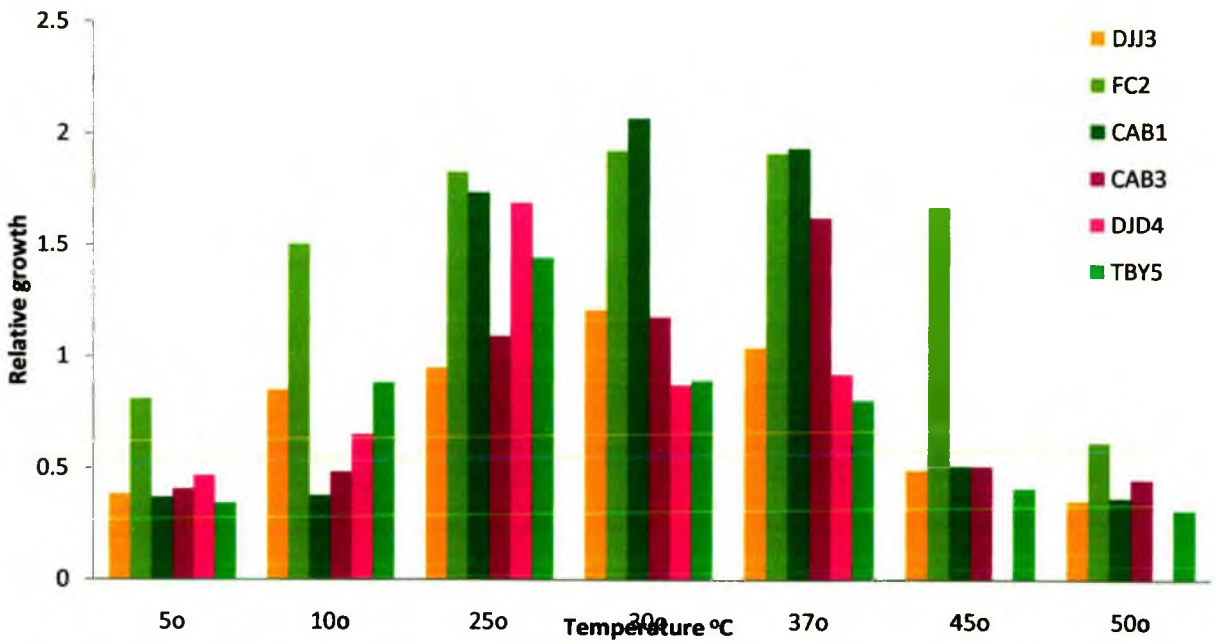

Fig 24 Growth response of aerobic isolates (DJJ3, FC2, CAB1, CAB3, DJD4 and TBY5) at different temperature (°C).

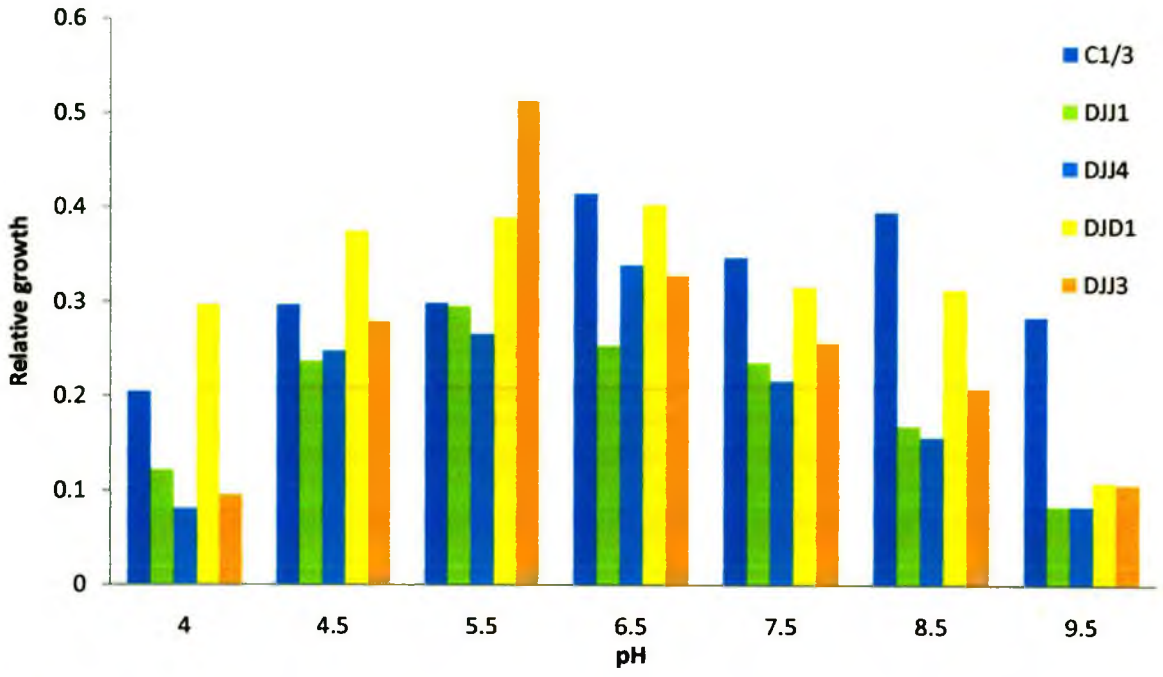

Fig 25 Growth response of aerobic isolates (C1/3, DJJ1, DJJ4, DJD1 and DJJ3) at different pH.

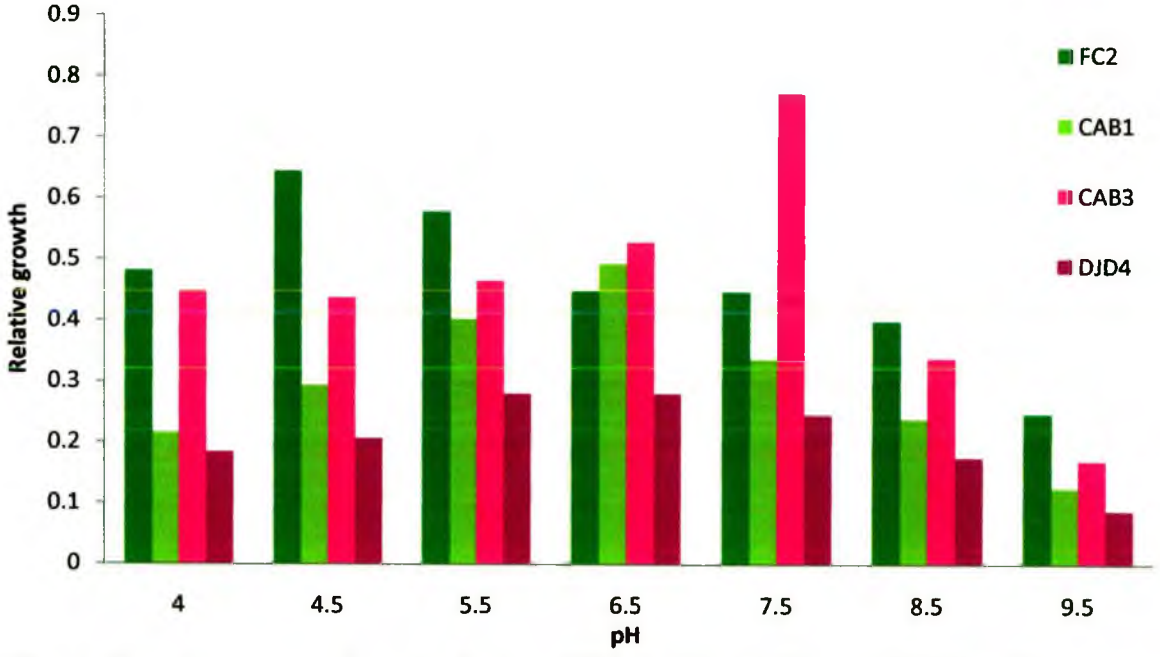

Fig 26 Growth response of aerobic isolates (FC2, CAB1, CAB3 and DJD4) at different pH.

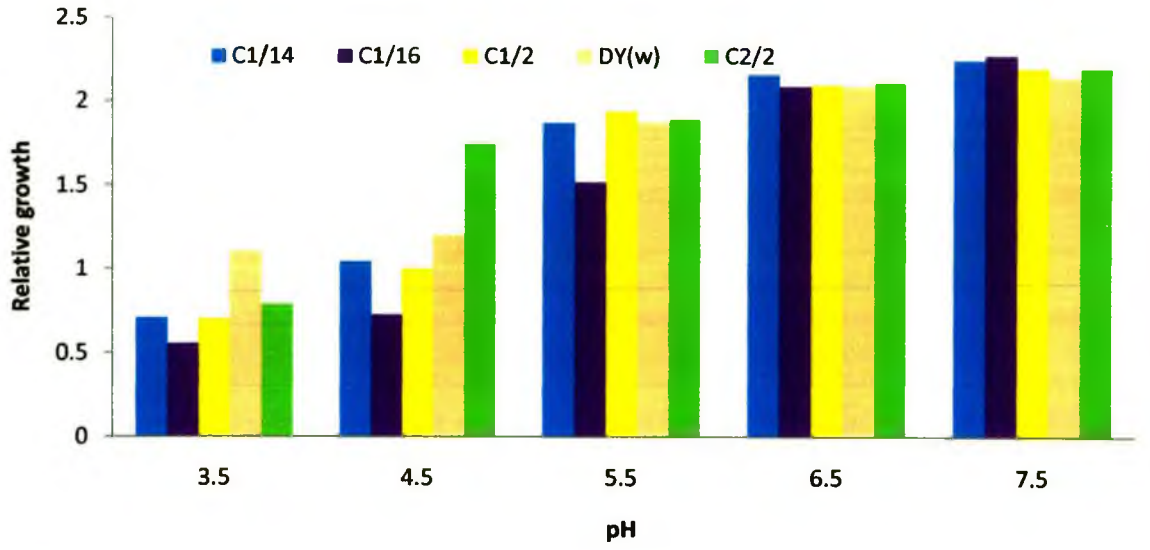

Fig 27 Growth response of anaerobic isolates (C1/14, C1/16, C1/2, DY(w) and C2/2) at different pH.

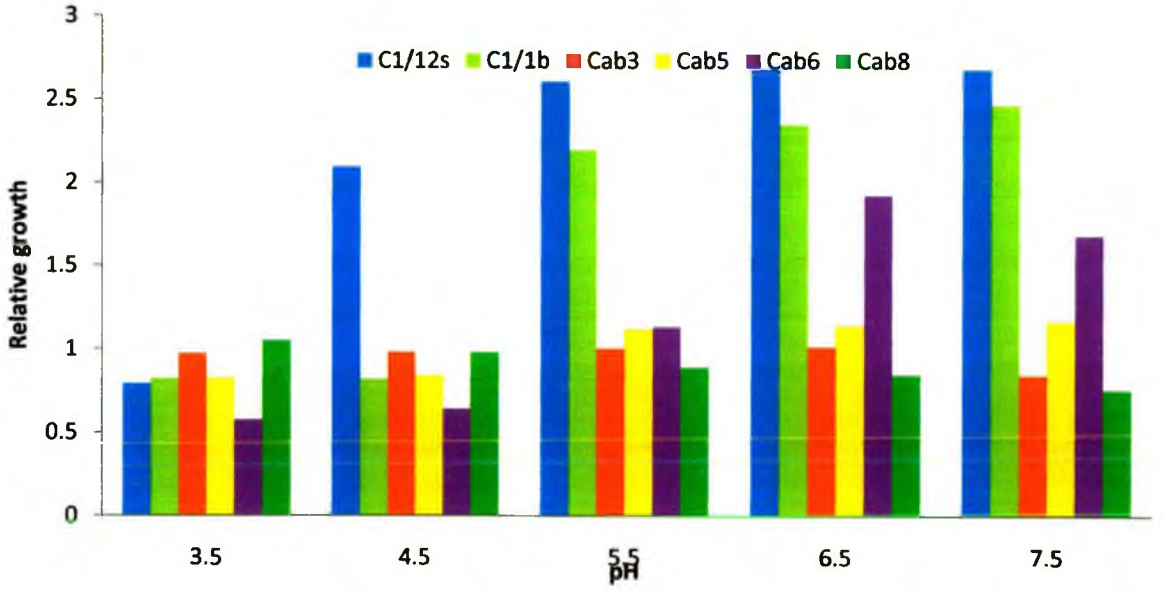

Fig 28 Growth response of anaerobic isolates (C1/14, C1/16, C1/2, DY(w) and C2/2) at different pH.

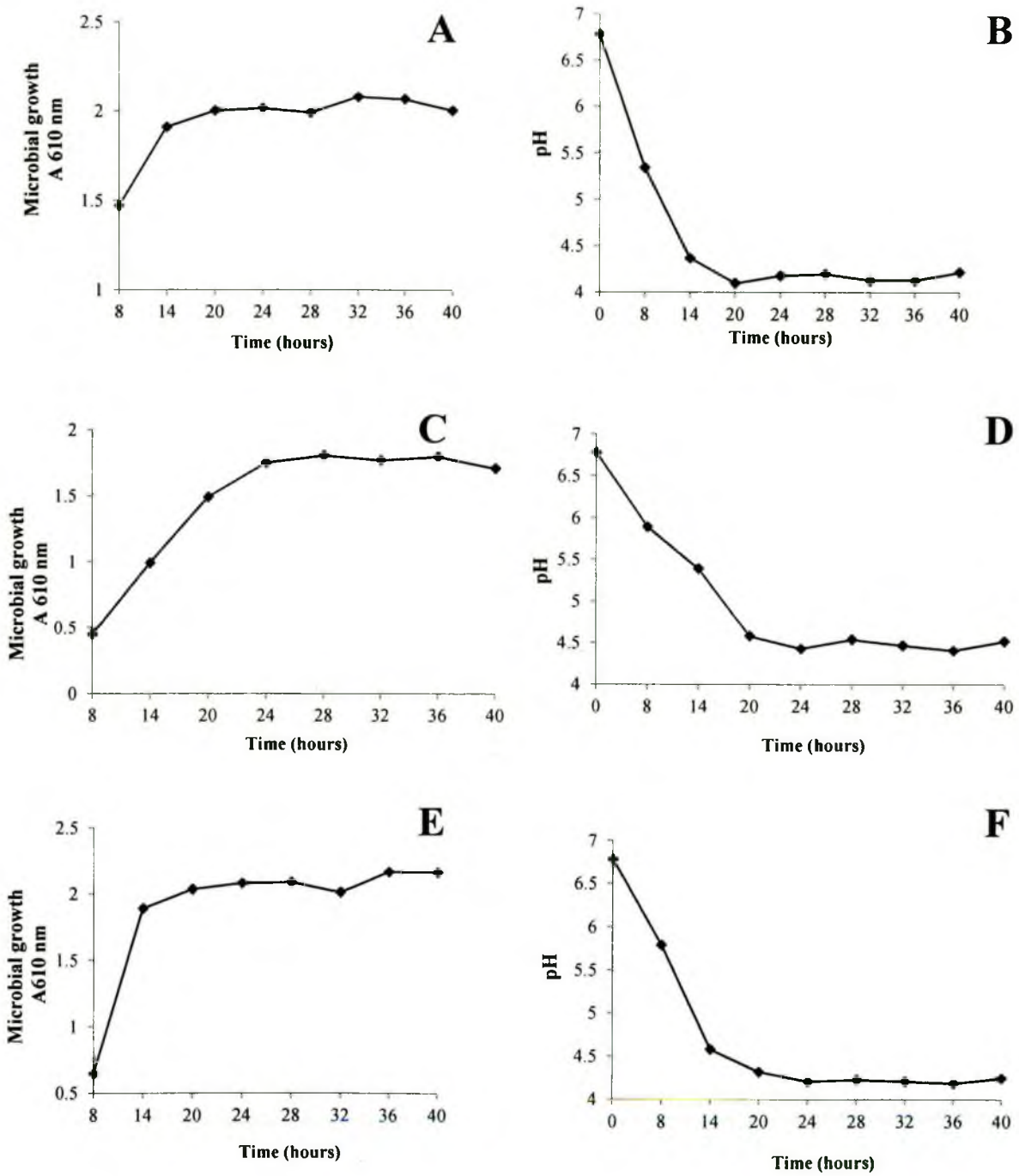

Fig 29. Growth curve of the selected anaerobic organisms and pH variations during incubation (A&B) C1/7, (C&D) C1/14 and (E&F) C1/12.

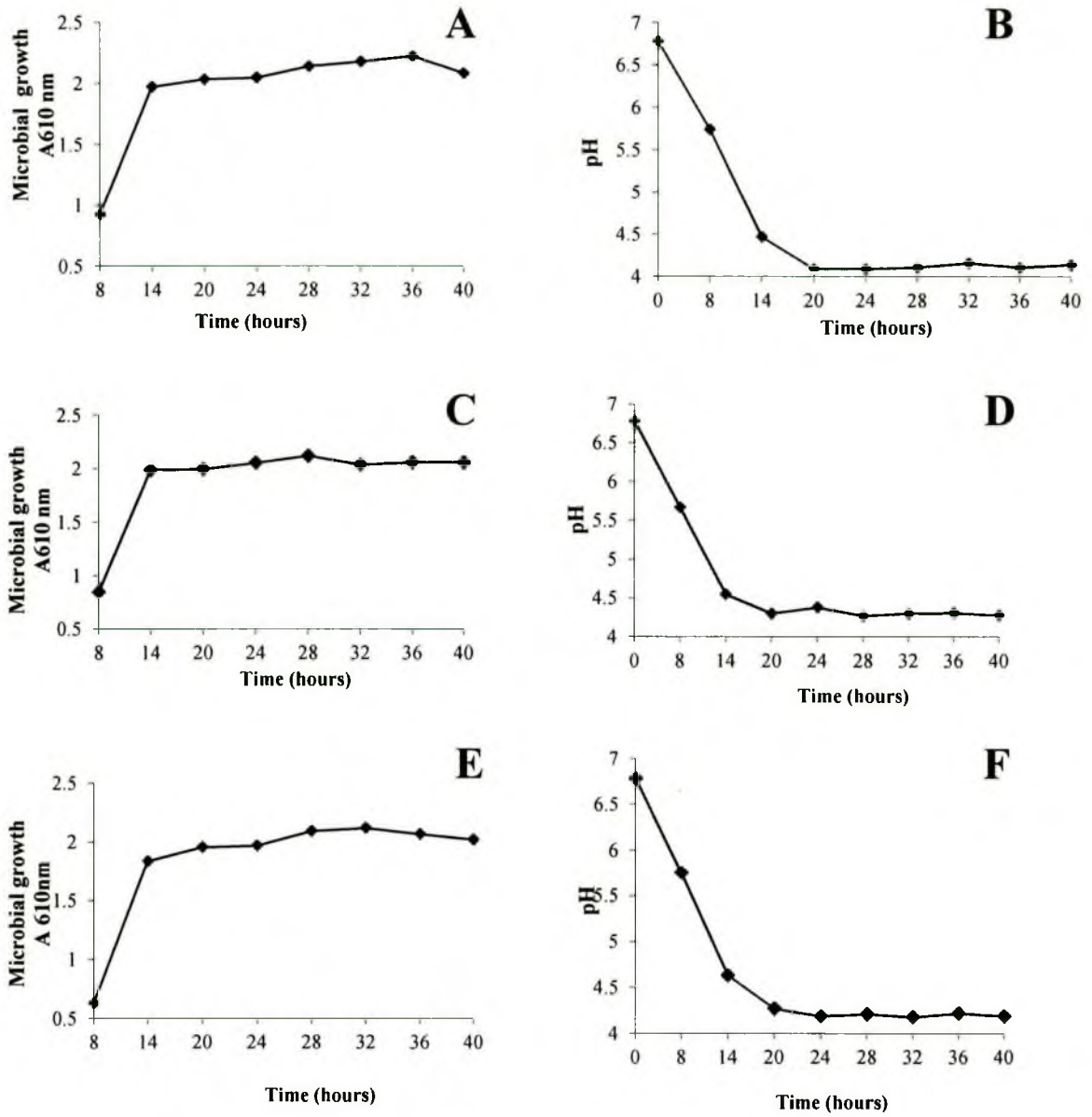

Fig 30. Growth curve of the selected anaerobic organisms and pH variations during incubation (A&B) C1/8(1), (C&D) C1/12a and (E&F) C1/10.

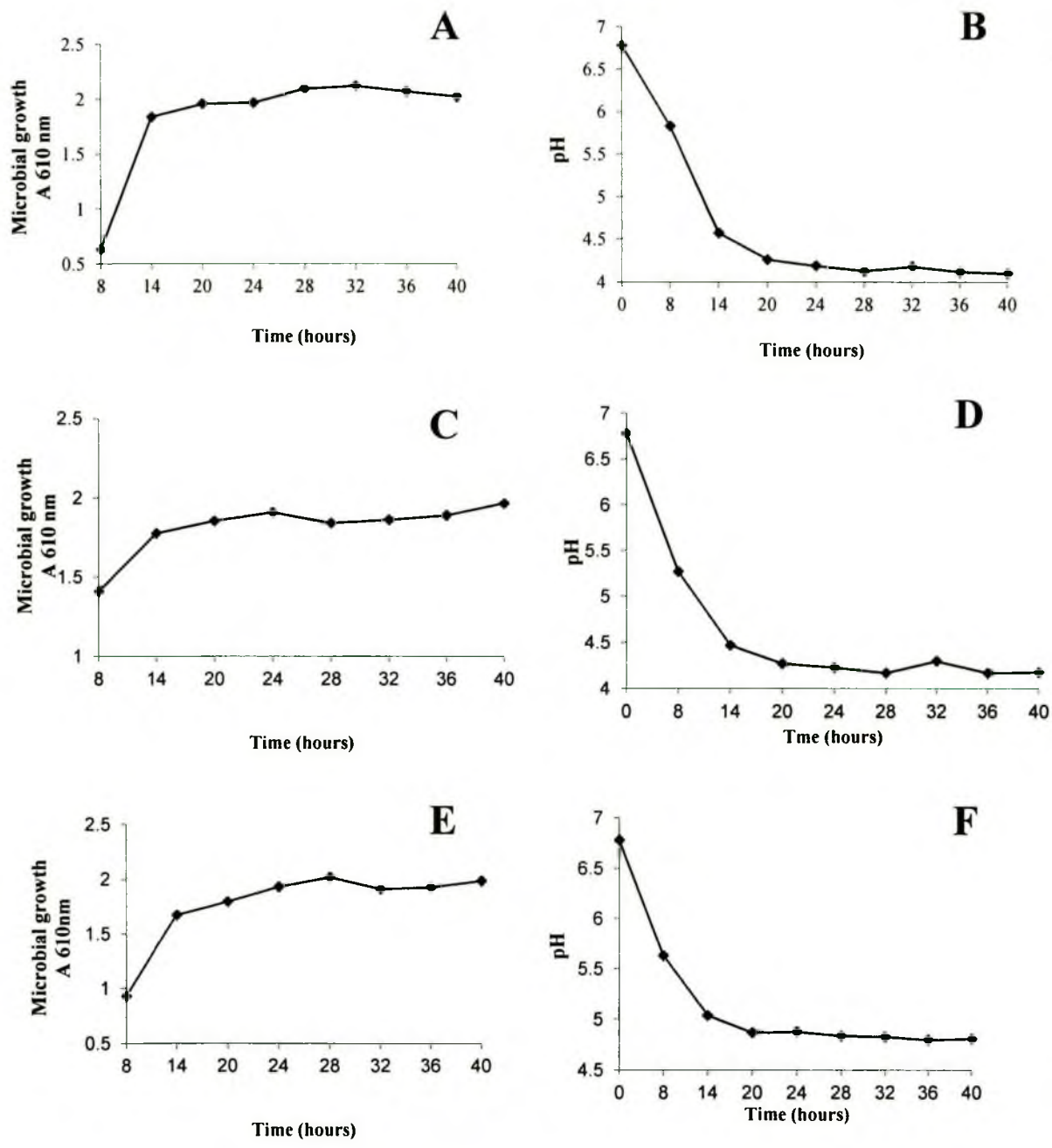

Fig 31. Growth curve of the selected anaerobic organisms and pH variations during incubation (A&B) C1/12b, (D&C) C1/16 and (E&F) DY(w).

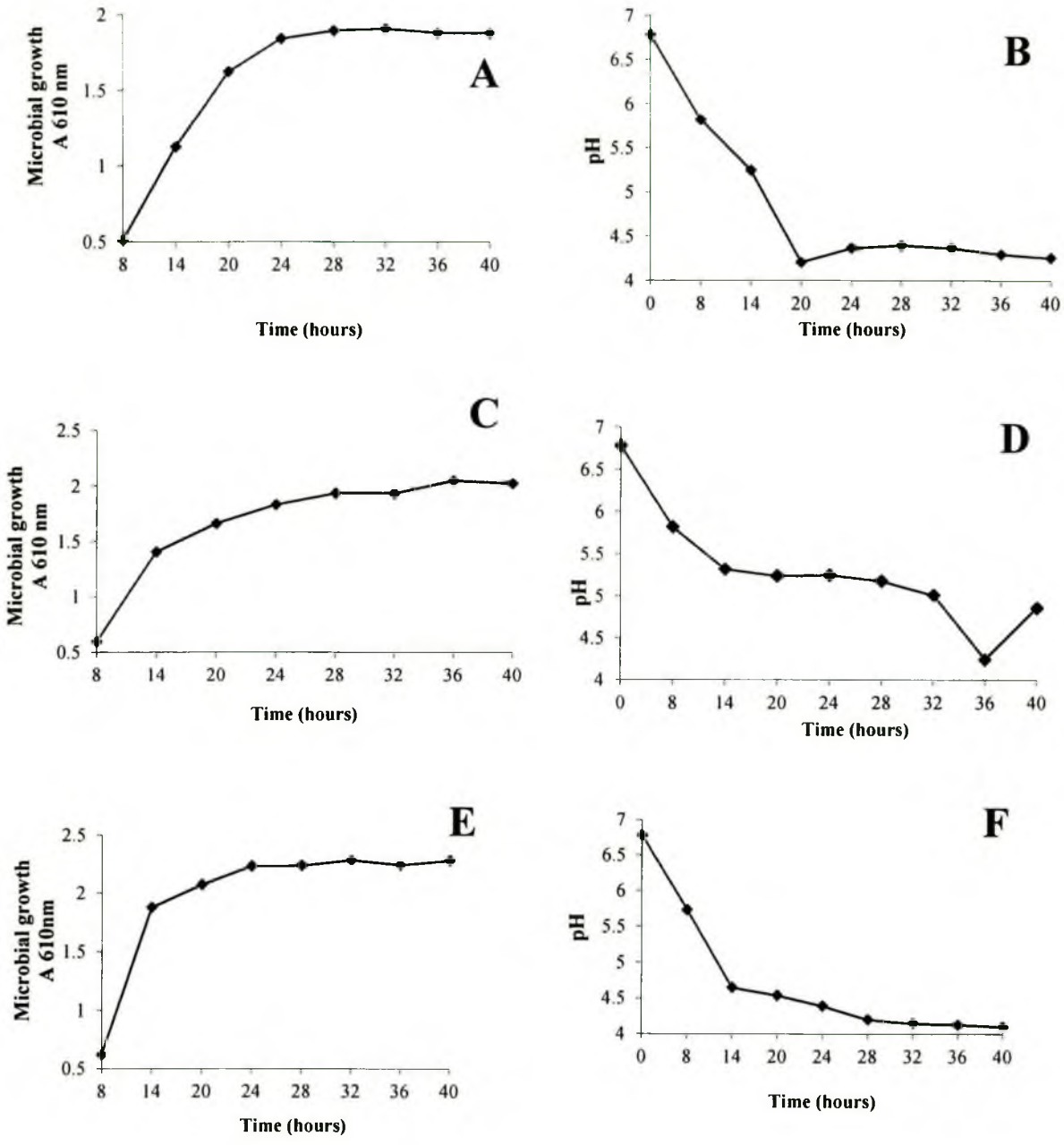

Fig 32. Growth curve of the selected anaerobic organisms and pH variations during incubation (A&B) C1/1b, (C&D) C2/6 and (E&F) Cab6

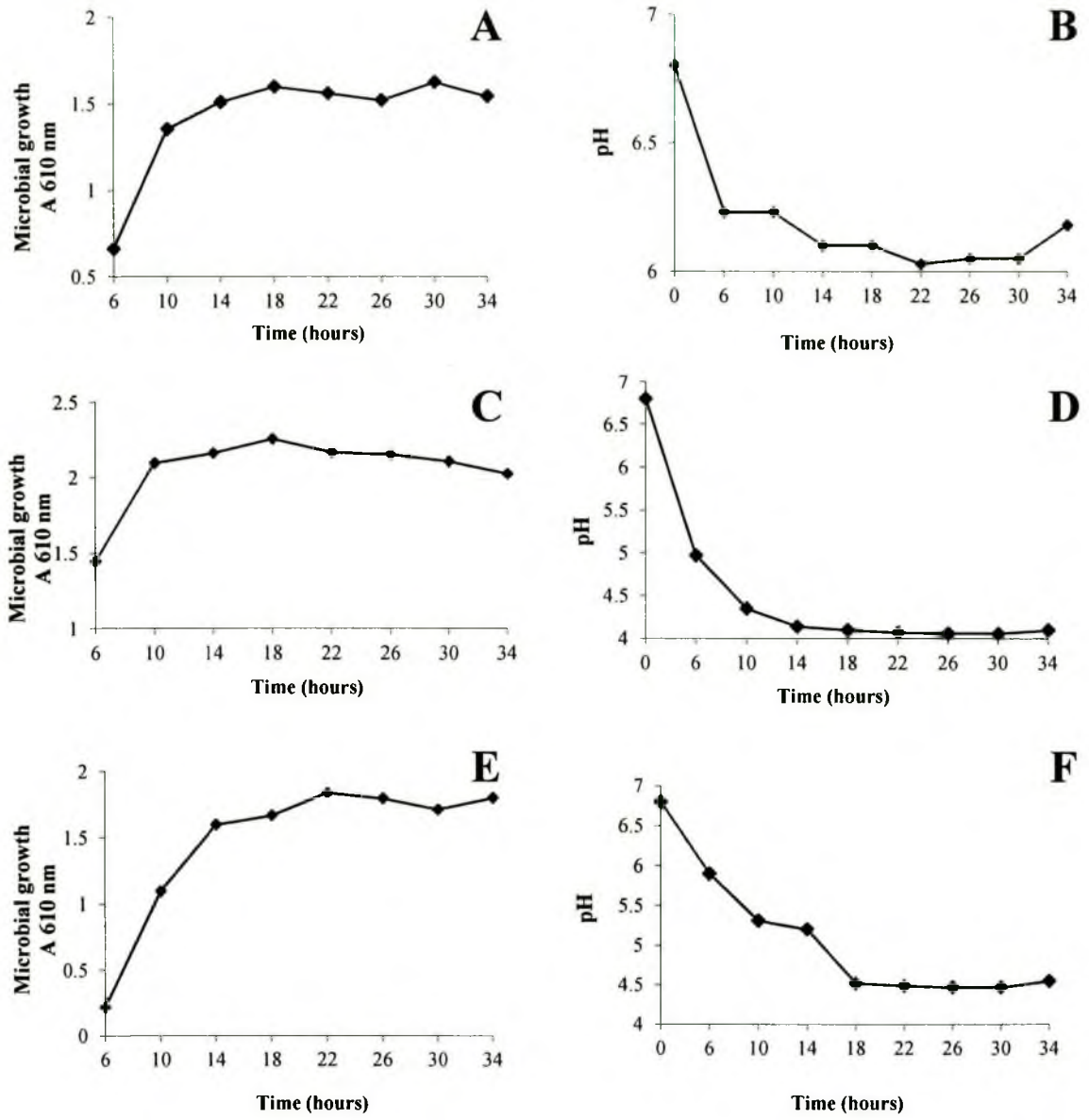

Fig. 33. Growth curve of the selected aerobic organisms and pH variations during incubation. (A &B) C3/4, (C&D) C1/3 and (E&F) DJJ4.

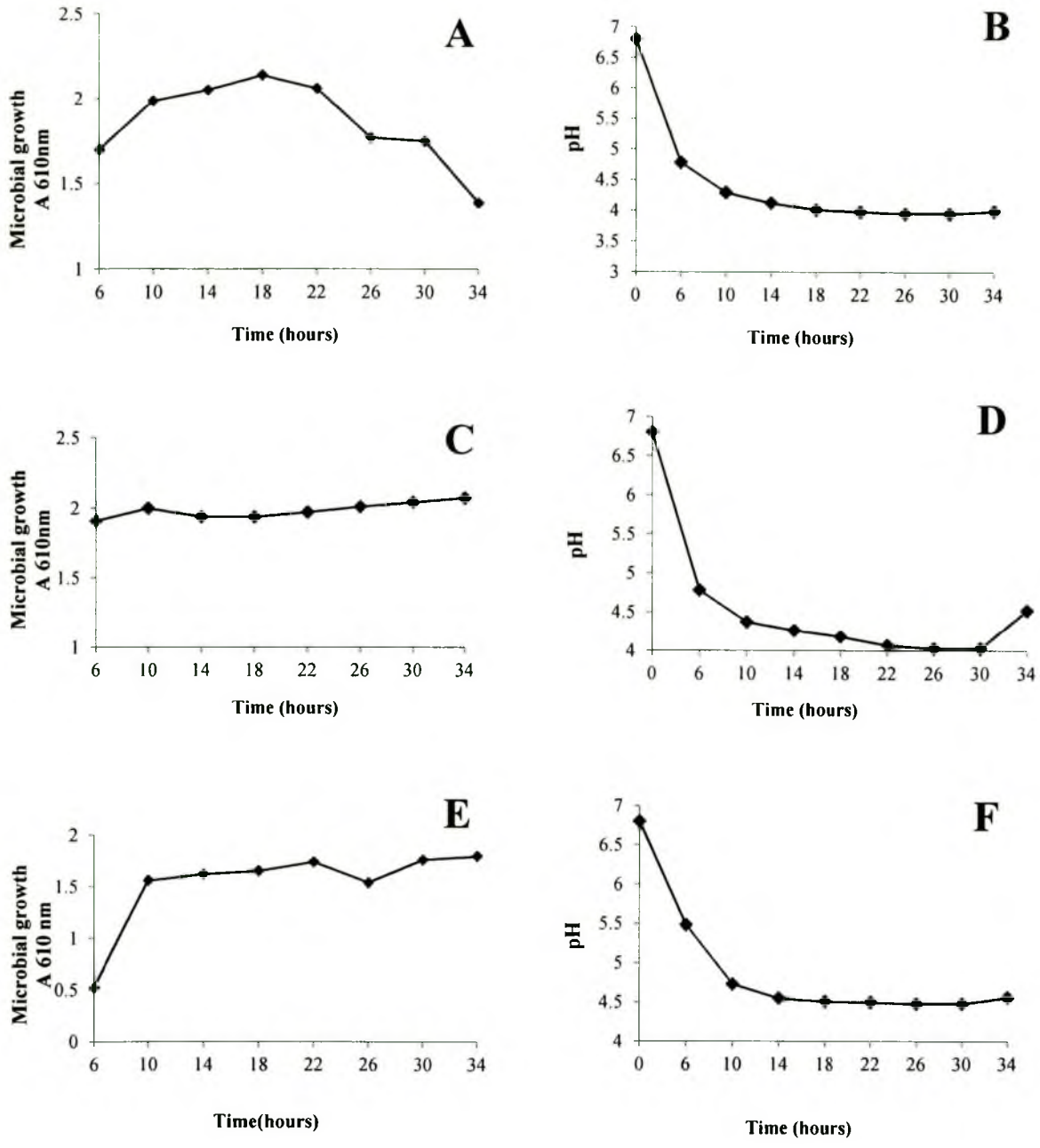

Fig. 34. Growth curve of the selected aerobic organisms and pH variations during incubation. (A&B) DJD1, (C&D) CAB1 and (E&D) DJD4.

Table 21: Provisional identification of the selected isolates (cont.)

Isolates Name	Provisional Identification
C3/4	*Lactobacillus kefir*
C3/2	*Lactobacillus kefir*
C3/1b	*Lactobacillus kefir*
C1/3	*Lactobacillus farciminis*
DJJ1	*Leuconostoc mesenteroides* subsp. *mesenteroides*
DJJ4	*Streptococcus lactis*
DJD1	*Lactobacillus vitulinus*
DJJ3	*Streptococcus parvulus*
FC2	*Leuconostoc mesenteroides* subsp. *cremoris*
CAB1	*Pediococcus urinaeequi*
CAB3	*Lactobacillus yamanashiensis*
DJD4	*Leuconostoc paramesenteroides*
TBY5	*Leuconostoc lactis*

Table 21: Provisional identification of the selected isolates (cont.)

Isolates Name	Provisional Identification
C1/7a	*L. casei* subsp. *pseudoplantarum*
C1/5a	*Lactobacillus coryniformis* subsp. *torquens*
C1/7	*Lactobacillus amaylophilus*
C1/14	*Not identified*
C1/12	*Lactobacillus delbrueckii* subsp. *bulgaricus*
C1/8(1)	*Lactobacillus casei* subsp. *pseudoplantarum*
C1/12a	*Lactobacillus jensenii*
C1/12s	*Lactobacillus farcimini*
C1/10	*Lactobacillus crispatus*
C1/12b	*Lactobacillus delbrueckii* subsp. *bulgaricus*
C1/16	*Lactobacillus plantarum*
DY(w)	*Lactobacillus kefir*
DY(w)3	*Lactobacillus sharpeae*
C1/2	*Lactobacillus bifermentans*
C2/2	*Lactobacillus bavaricus*

Table 21: Provisional identification of the selected isolates (cont.)

Isolates Name	Provisional Identification
C1/4	*Lactobacillus hilgardii*
GRK5	*Lactobacillus casei* subsp. *casei*
GRK6	*Lactobacillus coryniformis* subsp. *torquens*
GRK7	*Lactobacillus salivarius*
C1/8	*Lactobacillus maltaromicus*
C1/1	*Lactobacillus plantarum*
C1/1b	*Lactobacillus casei* subsp. *casei*
C2/6	*Lactobacillus salivarius*
Cab1	*Lactobacillus casei* subsp. *rhamnosus*
Cab2	*Lactobacillus crispatus*
Cab3	*Not identified*
Cab4	*Lactobacillus sharpeae*
Cab5	*Lactobacillus collinoides*
Cab6	*Lactobacillus crispatus*
Cab8	*Lactobacillus farcimini*

Applications

Antagonistic Activity of Probiotic Microorganisms

Probiotic microorganisms produce different types of antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins etc.

Organic acids are produced upon fermentation of sugar (Lactose, Glucose etc). Homofermentive microorganisms use the glycolytic pathway while homofermentive and heterofermentive microbes use pentose phosphate pathway to produce organic acids (acetic acid, Lactic acid, Propionic acid etc) (Blom and Mortvedl 1991; Caplice and fitzgerald 1999). Acetic acid is more inhibitory than lactic acid. Lactic acid has been shown to permeabilize the outer membrane of Gram-negative bacteria by liberation of its lipopolysaccharides, leading to loss of viability (Alakomi *et al.* 2000).

In the presence of flavoprotein oxidases and oxygen, bacteria are able to produce hydrogen peroxide (H_2O_2). The antimicrobial effect of hydrogen peroxide is attributed to a strong oxidizing effect on the bacterial cell. In addition, *L. crispatus* and *L. jensenii*, the most common lactobacilli in the female genital tract, have been shown to inhibit gonococci by producing H_2O_2 (St. Amant *et al.* 2002).

Carbon dioxide (CO_2) is mainly formed from heterolactic fermentation. It can directly create an anaerobic environment and is toxic to some aerobic food microorganisms. As CO_2 can effectively inhibit the growth of many food spoilage microbes, especially Gram-negative psychotropic bacteria (Daniel *et al.* 1985), the effect has been commercially applied to many refrigerated products as modified atmosphere packaging.

Probiotic microorganisms produce low molecular weight antimicrobial compounds such as Reuterin. It is produced by *L. reuteri,* an inhabitant of the gastrointestinal tract of humans

and animals. Reutarin is produced during stationary phase and anaerobically growth on a mixture of glucose and glycerol or glyceraldehydes (Axelsson *et al.* 1989). It has a wide antimicrobial spectrum affecting both Gram-positive and Gram-negative bacteria as well as yeasts, fungi and protozoa (Axelsson *et al.* 1989, Chung *et al.* 1989, Talarico and Dodrogosz 1989).

Bacteriocins are defined as proteinaceous, antibacterial substances synthesized by many different bacterial species in order to kill or inhibit growth of other bacteria. They are heterogeneous group of proteins that vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Bacteriocins are different from antibiotics. Gram-negative and Gram-positive, both types of bacteria can produce bacteriocins but comparatively wide spectrums of bacteriocins are produced by the former kind. Bacteriocins produced by Gram-positive bacteria usually LAB, inhibit strains of the same or closely related species (Héchard and Sahl 2002). There are three classes of bacteriocins produced by LAB-

ClassI: Lantibiotics (e.g. Lanthionine, β-methyllanthionine, Nisin etc).

ClassII:Small heat-stable peptides (e.g. Listeria-active or pediocin-like bacteriocin, two-peptide bacteriocins etc).

ClassIII: Large heat-labile proteins (includes large bactriocins whose mode of action is poorly described) (Klaenhammer 1993, Bauer and Dicks 2005; Héchard and Sahl 2002).

Bacteriocins of LAB have created attention for use as natural preservatives in food or in prbiotic applications. Probiotic bacteria can also produce antimicrobial substances such as

diacetyl acetaldehyde and ethanol. Gram-negative bacteria, yeasts and molds are more sensitive to diacetyl than Gram-positive bacteria.

Antagonistic activity assays:

Microorganisms, isolated under both aerobic and anaerobic environmental conditions, had provided their required growth conditions to observe their antagonistic activity. The inhibitory effects against pathogenic bacteria and fungi are enlisted in the table no. 22 and 23 respectively.

Giant colony and streak plate technique

The anti-microbial activities of microorganisms against selected pathogenic bacteria were assessed using a streak line (giant colony) method. It is the simplest procedure to determine the 'inhibition spectrum' of the isolates.

This procedure was applied to test antagonistic activity against pathogenic bacteria. For this purpose Modified Mueller-Hinton II agar (pH 6.8) were used.

Isolates were seeded in the middle of the agar plate using an inoculation loop. The plates were then incubated in aerobic or anaerobic environment at 37°C until adequate growth had occurred.

The test organisms were streaked for possible sensitivity to the isolates from the edges of the plates up to but not touching the giant colony. The plates were further incubated to allow growth of each test organism has been inhibited by antimicrobial substances in the vicinity of the test organism (Casida 1968, Hütt et al 2006). The antagonistic activity of the probiotic

microorganisms was estimated from the zone of inhibition (millimeter) (Mikelsaar et al 1987, Annuk et al. 2003).

Table 22: Pathogenic bacterial strains used for antimicrobial test:

Strain No.	Scientific Name of Pathogenic Bacteria	Strain No.	Scientific Name of Pathogenic Bacteria
1	*Staphylococcus aureus*	**M6**	*Enterobacter intermedium*
2	*Bordetella bronchiseptica*	**M7**	*Escherichia coli*
3	*Escherichia coli*	**N3**	*Bacillus fastidious*
6	*Micrococcus luteus*	**S4**	*Salmonella paratyphi*
7	*Staphylococcus epidermis*		

Four bacterial test organisms (M6, M7, N3 and S4) were taken from the collection of Microbiology laboratory of the Department of Botany Dhaka University (Md. Ali 2008) and the remaining five strains were collected from the Institution of Nutrition and Food Science, Dhaka University.

Table 23:Pathogenic Fungal strains used for antagonistic activity test:

Serial No.	Scientific name of pathogenic fungi
1	*Aspergillus niger*
2	*Aspeargillus fumigatus*
3	*Carvularia lunata*
4	*Fusarium moniliformae*
5	*Colletotrichum coffeanum*
6	*Trichoderma viride*

These pathogenic fungal strains were collected from the Pathology laboratory, Department of Botany, Dhaka University.

Sensitivity spectrum analyses by agar well diffusion method

Probiotic microorganisms were cultured in the modified MRS broth (pH 6.8) for 48 hrs at 37°C. Microbial cultures were centrifuged for 20 min at 12,000rpm and the supernatant fluids were frozen at 0°C. These supernatant fluids were used directly to observe inhibitory effects against pathogenic microbes.

In performing the sensitivity spectrum analyses by agar well diffusion method (BSI 1968), Potato dextrose agar (PDA) medium was used for fungi. Media were poured in Petri dishes and a hole was made in solidified media by cork borer under sterile condition. Then one drop of melted agar was poured into hole and allowed to solidify to make a base layer. After that specific amount of culture supernatant fluid (0.1 ml) was poured into the hole. Then plates were kept at low temperature (4°C) for 2-4 hours to allow maximum diffusion. During this time the test materials are dissolved and diffused out of the media. For this experiments two types of controls were maintained, one control was PDA medium with pathogenic microbes culture and another one contained pathogenic microbial growth with MRS medium in the hole.

The test fungal strains were inoculated at the side of hole and incubated at 30°C for 3days to allow maximum growth of the organisms.

Antagonistic activity of the culture supernatants of probiotic microorganisms

Preparation of supernatants: Modified MRS broth (pH6.8) was inoculated with freshly growing microorganisms and they were incubated at 37°C for 48hrs. Microbial cultures were

centrifuged for 20 min at 12,000 rpm and the supernatant was kept feozen. Before using supernatants, they were passed through the Whatman membrane filter (0.45μm pore size).

Method: A specific amount of filter sterilized supernatants were mixed thoroughly with the agar media (PDA and MH), so that the final concentration of the fluid was 0.2 ml per ml medium. For this experiment two types of controls were maintained- blank basal media and basal media with impregnated MRS broth. Pathogenic microorganisms were then streaked on the media and growth were visually measured and recorded.

Antagonistic activity at different pH:

Selective probiotic isolates were tested for their antagonistic activity against pathogenic bacteria and fungi at different pH. For this purpose, isolates were cultured in modified MRS broth of different pH (4.5, 6.5 and 8.5) at 30oC for 48 hrs. Then broth cultures were centrifuged for 20 min at 12,000rpm. Supernatant fluids were passed through membrane filter (45 μm) and mixed with the basal media (MH and PDA). Test bacteria were streaked on the MH medium and fungal culture blocks were inoculated on the PDA medium. Results were taken after 3 days and recorded

Result:

Antagonistic effects of probiotic microorganisms against pathogenic bacteria:

Inhibition spectrums of thirty-six isolates were studied employing giant colony technique against nine pathogenic bacterial strains. Majority of the isolates showed antagonistic effects against pathogens. Most of the anaerobic isolates showed strong antagonism against test

bacteria. Isolates C1/7a, C1/7, C1/12a, DY(w)3, C1/2, C2/2, C1/8, Cab1, Cab2 and Cab8 were very strongly active against pathogenic bacteria. (Table 24)

Antagonistic effects of probiotic microorganisms against pathogenic fungi:

Agar well diffusion method was used to observe antagonistic effect of the probiotic isolates against pathogenic fungi (Table 25).

Aerobic isolates C3/4, C3/2 and CAB3 inhibited the growth of *Aspergillus niger*. *A. fumigates* was inhibited by C1/3, DJJ1, DJD1, DJD4 and TYB5. *Fusarium moniliformae* was inhibited by C2/3 and DJJ3. C3/2, C3/4 and DJJ3 were active against *Trichodearma viride*. . DJJ3 showed antagonistic activity against four fungi except *A. niger* and *A. fumigates*.

Anaerobic isolates C1/7a, C1/7, C1/12, C1/12a, DY(w), GRK7 and C2/6 were strongly active against six pathogenic fungi. C1/4, C1/8 and Cab5 did not have any antimicrobial activity against test fungal strains.

Effect of culture filtrates on the growth of pathogenic bacteria:

Among anaerobic probiotic isolates, C1/5a, DY(w)3, C1/2, C2/2, C1/8, Cab2 and Cab8 had strong antagonistic activity against test bacteria and CAB3 alone aerobic isolate had strong antagonistic effect (Table 26 and Figure 35-36).

Effect of culture filtrates on the growth of pathogenic fungi:

Culture filtrates of probiotic isolates were mixed with basal medium and pathogenic fungi were inoculated to observe their inhibitory effects on the fungi. Most of the isolates showed

antimicrobial effects on the growth and sporulation of the fungi. DY(w), C1/8 and C2/6 inhibited growth and sporulation of the six test fungi. But C1/4 had no effect on these six pathogenic microbes (Table 27 and Figure 37-42).

Effect of culture filtrates of different pH on the growth of pathogenic microorganisms:

Isolate no. DY(w), C2/6 and C1/8 were cultured in modified MRS medium with optimum incubation period at different pH (4.5, 6.5 and 8.5) to observe their antagonistic effects on the pathogenic microbes (Table 28 and 29).

Table 24: Antagonistic activity test of probiotic microorganisms employing Giant colony technique in MH medium (Cont.):

Aerobic Isolates No.	Inhibition spectrum of the selected isolates against pathogenic bacteria (measured in mm)								
	PATHOGENIC BACTERIA								
	1	**2**	**3**	**6**	**7**	**M6**	**M7**	**N3**	**S3**
C3/4	-	-	GR	-	GR	GR	GR	-	GR
C3/2	-	GR	GR	-	GR	GR	GR	-	-
C3/1b	-	-	-	-	-	-	-	-	-
C1/3	18	NG	16	18	-	-	-	GR	GR
DJD 1	-	-	19	22	4	6	3	11	7
FC2	18	9	-	15	-	-	-	-	-
Anaerobic Isolates No.	PATHOGENIC BACTERIA								
	1	**2**	**3**	**6**	**7**	**M6**	**M7**	**N3**	**S3**
C1/7a	24	21	NG	NG	22	NG	NG	NG	21
C1/5a	GR	22	19	19	23	17	20	21	16
C1/7	21	22	22	24	25	GR	24	19	21
C1/14	NG	12	NG	10	16	18	NG	NG	12
C1/12	GR	GR	14	15	13	11	GR	15	18
C1/8(1)	12	16	15	19	NG	13	11	16	10
C1/12a	23	23	NG	22	NG	NG	NG	NG	21
C1/12s	21	21	NG	NG	NG	NG	20	NG	16
C1/10	-	-	GR	GR	NG	NG	-	16	GR
C1/12b	18	14	NG	NG	NG	15	NG	12	GR
C1/16	GR	13	22	20	16	GR	GR	12	GR

'NG'- no growth, 'GR'- growth reduced, '–'- no inhibition

Table 24: Antagonistic activity test of probiotic microorganisms employing Giant colony technique in MH medium:

Anaerobic Isolates No.	Inhibition spectrum of the selected isolates against pathogenic bacteria (measured in mm)								
	PATHOGENIC BACTERIA								
	1	**2**	**3**	**6**	**7**	**M6**	**M7**	**N3**	**S3**
DY(w)	16	NG	21	20	19	17	13	GR	11
DY(w)3	NG	23	NG	NG	NG	NG	NG	22	19
C1/2	NG	NG	NG	21	NG	NG	NG	NG	NG
C2/2	NG	NG	NG	NG	NG	NG	NG	NG	NG
C1/4	21	-	NG	16	20	NG	21	NG	12
GRK5	12	14	NG	-	NG	11	NG	16	GR
GRK6	23	12	NG	22	NG	21	21	20	15
GRK7	GR	12	10	14	16	11	-	-	12
C1/8	NG	21	NG	NG	NG	NG	NG	NG	NG
C1/1	22	19	NG	18	15	NG	NG	NG	16
C2/6	20	20	23	12	NG	12	19	13	-
C1/1b	GR	11	10	GR	19	8	11	-	-
Cab1	22	24	NG	NG	NG	NG	21	NG	19
Cab2	NG	NG	NG	NG	NG	NG	NG	NG	23
Cab3	22	21	NG	23	NG	19	16	21	14
Cab4	21	20	13	GR	NG	NG	NG	GR	9
Cab5	18	14	21	NG	NG	22	22	NG	16
Cab6	18	21	NG	13	NG	NG	9	NG	16
Cab8	21	20	NG	NG	NG	NG	NG	NG	19

'NG'- no growth, 'GR'- growth reduced, '–'- no inhibition

Table 25: Sensitivity spectrum estimation by using seeded MRS broth against pathogenic fungi (Cont.)

Aerobic Isolates No.	PATHOGENIC FUNGAL STRAINS					
	Aspergillus niger	*Aspergillus fumigatus*	*Carvularia lunata*	*Fusarium moniliformae*	*Colletotrichum coffeanum*	*Trichoderma viride*
C3/4	+	-	+	-	-	+
C3/2	+	-	+	+	-	+
C3/1b	-	-	-	-	+	-
C1/3	-	+	-	-	-	-
DJD 1	-	+	+	-	-	-
DJJ4	-	-	-	-	-	+
DJJ1	-	+	-	-	-	-
DJJ3	-	-	+	+	+	+
FC2	-	-	-	-	+	-
CAB1	-	-	+	-	+	-
CAB3	+	-	+	-	+	-
DJD4	-	+	+	-	+	-
TBY5	-	+	+	-	-	+

'+' antagonistic activity present; '-'antagonistic activity absent

Table 25: Sensitivity spectrum estimation by using seeded MRS broth against pathogenic fungi (Cont.)

Anaerobic Isolates no.	PATHOGENIC FUNGAL STRAINS					
	Aspergillus niger	*Aspergillus fumigatus*	*Carvularia lunata*	*Fusarium moniliformae*	*Colletotrichum coffeanum*	*Trichoderma viride*
C1/7a	+	+	+	+	+	+
C1/5a	-	+	+	+	+	-
C1/7	-	+	+	+	+	-
C1/14	-	-	+	-	+	-
C1/12	+	+	+	+	+	+
C1/8(1)	-	+	+	+	+	+
C1/12a	+	+	+	+	+	+
C1/12s	+	+	+	+	-	-
C1/10	-	+	+	+	+	+
C1/12b	-	-	+	+	+	+
C1/16	-	+	+	+	+	-
DY(w)	+	+	+	+	+	+
DY(w)3	-	-	+	-	+	+
C1/2	-	+	-	+	+	+
C2/2	-	-	-	+	+	+

'+' antagonistic activity present; '-'antagonistic activity absent

Table 25: Sensitivity spectrum estimation by using seeded MRS broth against Pathogenic fungi

Anaerobic Isolates no.	PATHOGENIC FUNGAL STRAINS					
	Aspergillus niger	*Aspergillus fumigatus*	*Carvularia lunata*	*Fusarium moniliformae*	*Colletotrichum coffeanum*	*Trichoderma viride*
C1/4	-	-	-	-	-	-
GRK5	-	-	+	-	+	+
GRK6	-	-	-	-	-	-
GRK7	+	+	+	+	+	+
C1/8	-	-	-	-	-	-
C1/1	-	-	+	+	+	+
C2/6	+	+	+	+	+	+
C1/1b	-	+	+	+	+	+
Cab1	-	-	+	+	+	+
Cab2	-	+	+	+	+	+
Cab3	-	-	+	+	+	+
Cab4	-	-	+	-	+	+
Cab5	-	-	-	-	-	-
Cab6	-	-	+	+	+	+
Cab8	-	-	+	+	+	+

'+' antagonistic activity present; '-'antagonistic activity absent

Table 26: Effects of culture filtrate of probiotic microbes on the growth of Pathogenic bacteria (Cont.):

Control	PATHOGENIC BACTERIA								
	1	**2**	**3**	**6**	**7**	**M6**	**M7**	**N3**	**S3**
MH	4	-	5	3	3	4.5	4	3	-
MH+MRS	5	3	4	4	5	5	5	5	3
Extracts of aerobic isolates									
C1/3	0.2	-	-	0.2	-	-	-	0.2	-
DJD 1	1	1	1	0.5	-	-	0.5	0.5	1.5
DJJ1	1.5	2	0.5	2.5	0.5	1	0.5	1	1
DJJ3	1.5	2.5	1	1	2.5	2	2.5	4	2
FC2	1	0.5	1.5	1	1.5	1	0.5	2	0.5
CAB1	0.5	2	0.5	1	1	2	1	0.5	1.5
CAB3	0.5	-	-	-	-	-	-	-	-
DJD4	0.5	1	1.5	0.5	-	1	-	0.5	1.5
TBY5	0.5	1	0.5	1.5	1	1.5	1	0.5	2
Extracts of anaerobic isolates									
C1/7a	-	0.5	-	-	0.1	-	-	-	0.5
C1/5a	-	-	-	-	-	-	-	-	-
C1/7	0.5	0.5	0.5	-	-	-	0.5	0.5	0.5
C1/14	-	1	-	0.5	0.5	1	-	-	3
C1/12	1	0.5	1	1	0.5	0.5	-	0.5	0.5
C1/8(1)	1	0.5	1	1	-	1	0.5	0.5	1
C1/12a	-	-	-	0.2	-	-	-	-	0.2

'-' No growth; '5' maximum growth; '0.1' minimum growth

Table 26: Effects of culture filtrate of probiotic microbes on the growth of pathogenic bacteria

Extracts of anaerobic isolates	PATHOGENIC BACTERIA								
	1	**2**	**3**	**6**	**7**	**M6**	**M7**	**N3**	**S3**
C1/12s	1	1	-	-	-	-	0.2	-	1
C1/10	0.5	0.5	1	1	1	-	0.5	0.5	0.5
C1/12b	1.5	1.5	-	-	-	0.5	-	1	1
C1/16	1	0.5	1	1	0.5	1	1	1	1
DY(w)	1	-	0.5	1	0.5	0.5	0.5	1	0.5
DY(w)3	-	1	-	-	-	-	-	0.5	0.5
C1/2	-	-	-	0.2	-	-	-	-	-
C2/2	-	-	-	-	-	-	-	-	-
C1/4	0.2	1.5	-	1	0.3	-	0.2	-	1.5
GRK5	0.5	2	-	1	-	1	-	0.5	2.5
GRK6	0.5	1.5	-	0.5	-	0.2	0.2	0.2	1
GRK7	2	1	1	1	1	1	1	1	1
C1/8	-	0.5	-	-	-	-	-	-	-
C1/1	0.5	2	-	0.5	0.5	-	-	-	1
C2/6	1	1	0.5	2	-	2	1	2	2
C1/1b	1	2	1	2	0.5	1	1	1	2
Cab1	0.3	0.5	-	-	-	-	0.3	-	0.5
Cab2	-	-	-	-	-	-	-	-	0.1
Cab3	1.5	1	-	0.5	-	0.5	0.5	0.5	1
Cab4	1.5	0.5	0.5	0.5	-	-	-	0.5	1
Cab5	0.5	0.3	0.1	-	-	0.2	0.2	-	0.3
Cab6	0.5	1.5	-	0.5	-	-	0.5	-	1
Cab8	0.5	0.5	-	-	-	-	-	-	0.5

'-' No growth; '5' maximum growth; '0.1' minimum growth

Table 27: Effects of culture filtrate of probiotic microbes on the growth of pathogenic fungi (cont.):

Control	PATHOGENIC FUNGAL STRAINS [Colony diameter (mm)]					
	Aspergillus niger	***Aspergillus fumigatus***	***Carvularia lunata***	***Fusarium moniliformae***	***Colletotrichum coffeanum***	***Trichoderma viride***
PDA	90	33	51	41	38	90
PDA+MRS	90	34	42	28	23	85
Extracts of anaerobic isolates						
C1/7a	GR	GR	43	GR	GR	GR Trace sporulation
C1/5a	58	17	20	19	16	58
C1/7	54	20	16	20	15	73
C1/14	72	-	16	-	21	55 sporulation reduced
C1/12	55	13	No growth Trace sporulation	10	5 Sporulation reduced	20
C1/8(1)	69	16	NG NS	No growth sporulation at surface	Trace amount of sporulation and growth present	34
C1/12a	GR	24	NG	NG	27	GR Trace sporulation
C1/12s	GR	GR	24	GR	18	-
C1/10	90	15	14	12	5	47
C1/12b	-	-	18	GR	20	Sporulation reduced
C1/16	65	22	17	19	21	69
DY(w)	8 small amount of sporulation	NG NS	NG NS	NG NS	NG NS	NG NS
DY(w)3	-	58	15	-	23	Sporulation reduced

'-' no effect, GR growth reduced; NG No growth; NS no sporulation

Table 27: Effects of culture filtrate of probiotic microbes on the growth of pathogenic fungi

Extracts of anaerobic isolates	PATHOGENIC FUNGAL STRAINS [Colony diameter (mm)]					
	Aspergillus niger	*Aspergillus fumigatus*	*Carvularia lunata*	*Fusarium moniliformae*	*Colletotrichum coffeanum*	*Trichoderma viride*
C1/2	-	GR	18	GR	GR	GR Trace sporulation
C2/2	-	-	16	GR	GR	GR Trace sporulation
C1/4	-	-	-	-	-	-
GRK5	61	-	18	-	14	Sporulation reduced
GRK6	-	-	35	21	23	-
GRK7	46	18	11	11	12 small amount of sporulation	34
C1/8	NG	NG	NG	NG	NG	NG
C1/1	-	-	7	12	13	Sporulation reduced
C2/6	6 small amount of sporulation	NG NS	NG Trace sporulation	NG Trace sporulation	NG NS	NG NS
C1/1b	46	14	NG Trace sporulation	9	5 Trace sporulation	21
Cab1	-	-	16	12	GR	GR Trace sporulation
Cab2	-	GR	G	GR	GR	GR Trace sporulation
Cab3	-	-	20	24	15	Reduced Sporulation
Cab4	-	43	18	-	17	Sporulation reduced
Cab5	-	-	24	GR	-	-
Cab6	-	49	16	GR	14	Sporulation reduced
Cab8	-	-	13	NG	-	45

'-' no effect, GR growth reduced; NG No growth; NS no sporulation

Table 28: Antagonistic activity of the culture filtrates of selected probiotic isolates of different pH against pathogenic bacteria

Test Bacteria	Cont. MH	Cont. MH+MRS			DY(w)			C2/6		
		MRS 4.5	MRS 6.5	MRS 8.5	4.5	6.5	8.5	4.5	6.5	8.5
1	3	1	2	4	1	-	1	-	-	1.5
2	-	1	2	2	2	-	1.5	1.5	-	1.5
3	3	1	2	3	1	-	2	-	-	3
6	2	1	3	3	2	-	1	-	-	2
7	4	-	5	4	-	-	-	-	-	3
M6	2	2	2	3	1	-	2	-	-	2
M7	2	3	3	3	2	-	3	-	-	3
N3	4	5	5	5	3	-	2	-	-	3
	-	2	3	2	2	-	1	2	1	1.5

- No growth; 0-5 minimum to maximum growth

Table 29: Antagonistic activity of the culture filtrates of selected probiotic isolates of different pH against pathogenic fungi

		PATHOGENIC FUNGAL STRAINS				
	pH	*Aspergillus niger*	*Aspergillus fumigates*	*Carvularia lunata*	*Fusarium moniliformae*	*Trichoderma viride*
Control PDA	5.5	+	+	+	+	+
Control PDA +MRS	4.5	+	+	+	+	+
	6.5	+	+	+	+	+
	8.5	+	+	+	+	+
DY(w)	4.5	-	GR	GR	+	-
	6.5	-	+	GR	+	-
	8.5	+	+	GR	GR	+
C2/6	4.5	-	GR	GR	GR	-
	6.5	-	GR	GR	GR	-
	8.5	+	GR	GR	GR	+
C1/8	4.5	GR	GR	GR	GR	GR
	6.5	GR	GR	GR	GR	GR
	8.5	-	-	-	-	-

'+' growth present; '-' growth absent; GR growth reduced

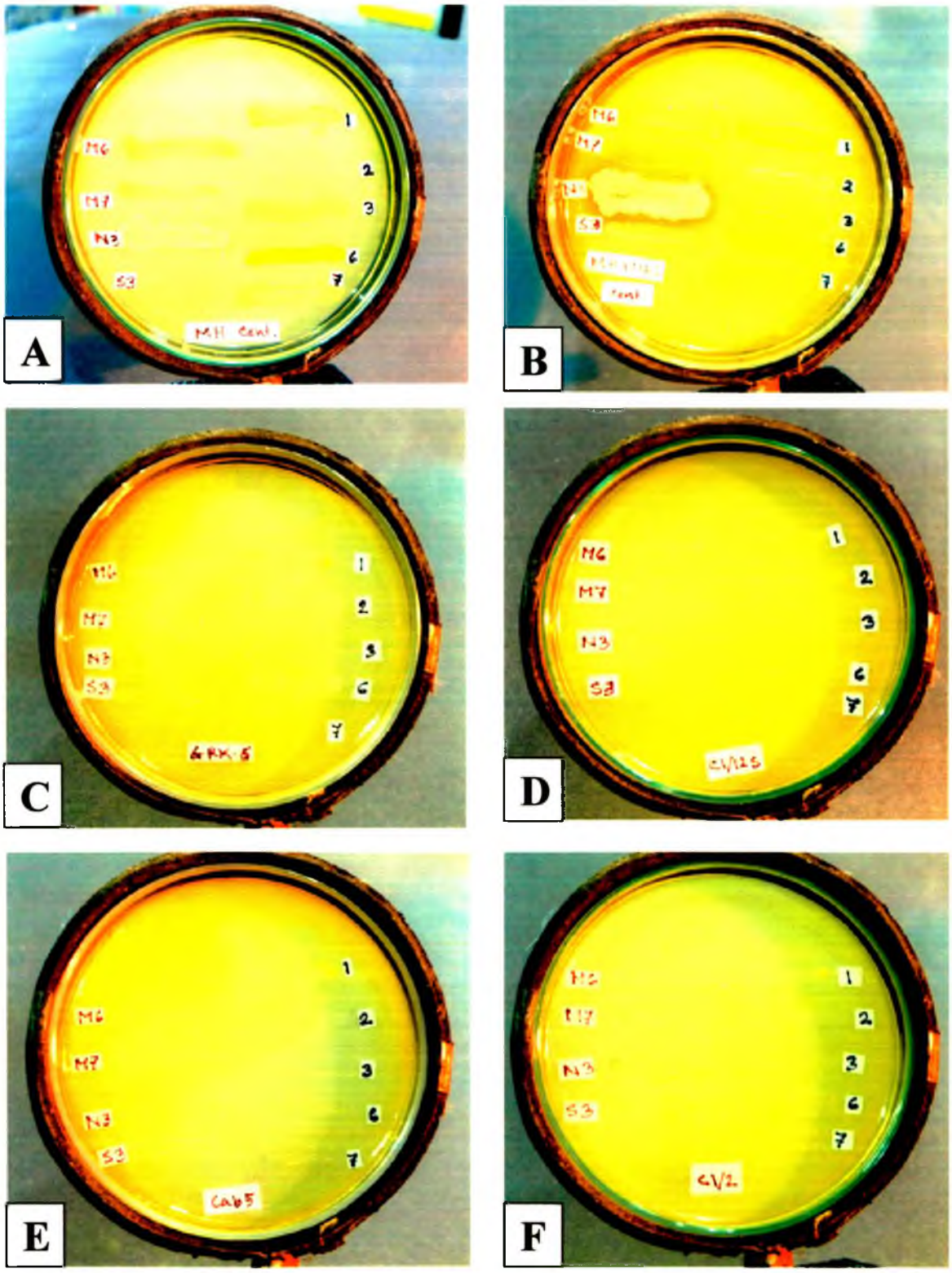

Fig. 35. Antimicrobial activity of the culture filtrate of probiotic isolates against pathogenic bacteria (A-F).

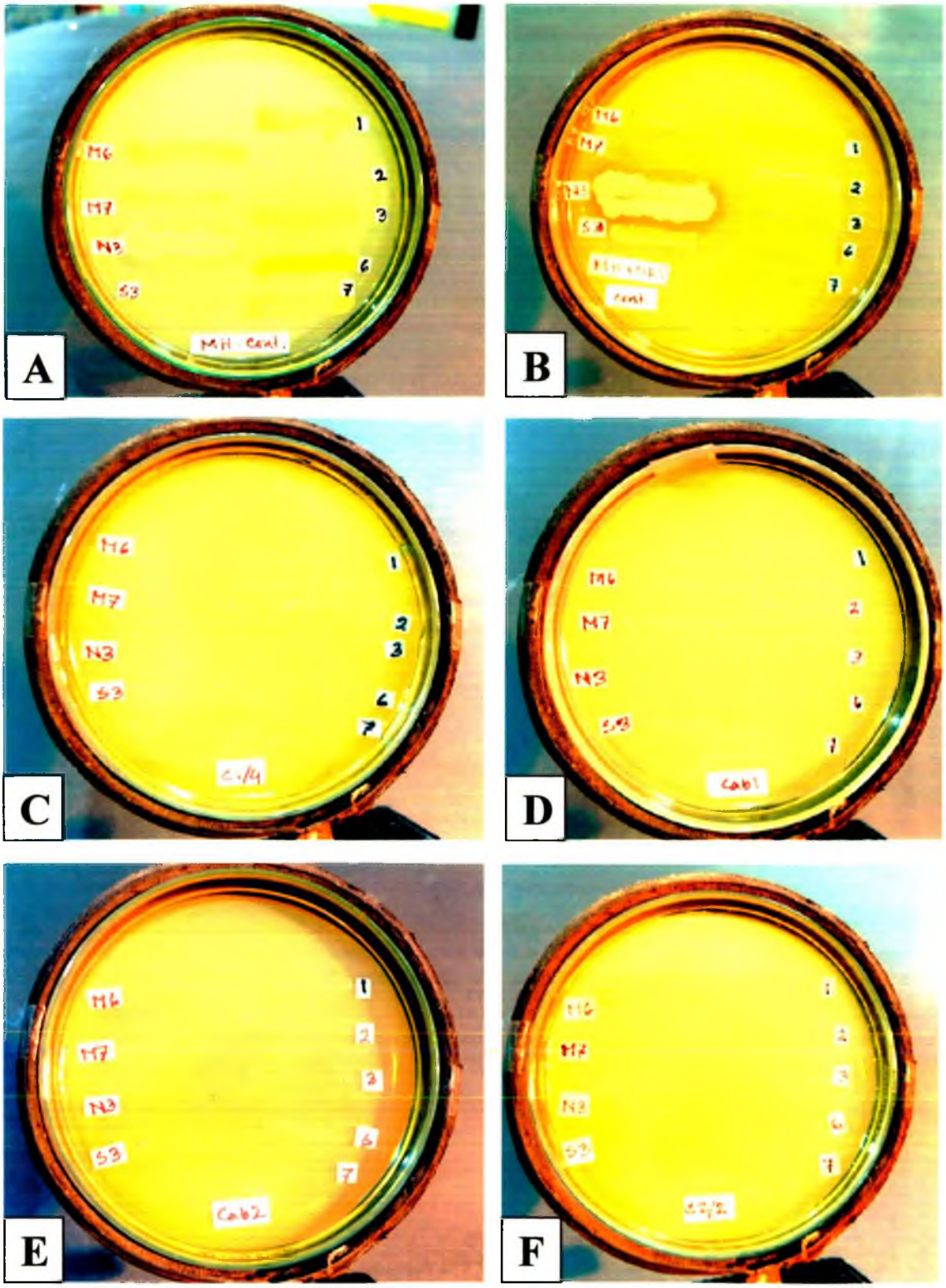

Fig. 36. Antimicrobial activity of the culture filtrate of probiotic isolates against pathogenic bacteria (A-F).

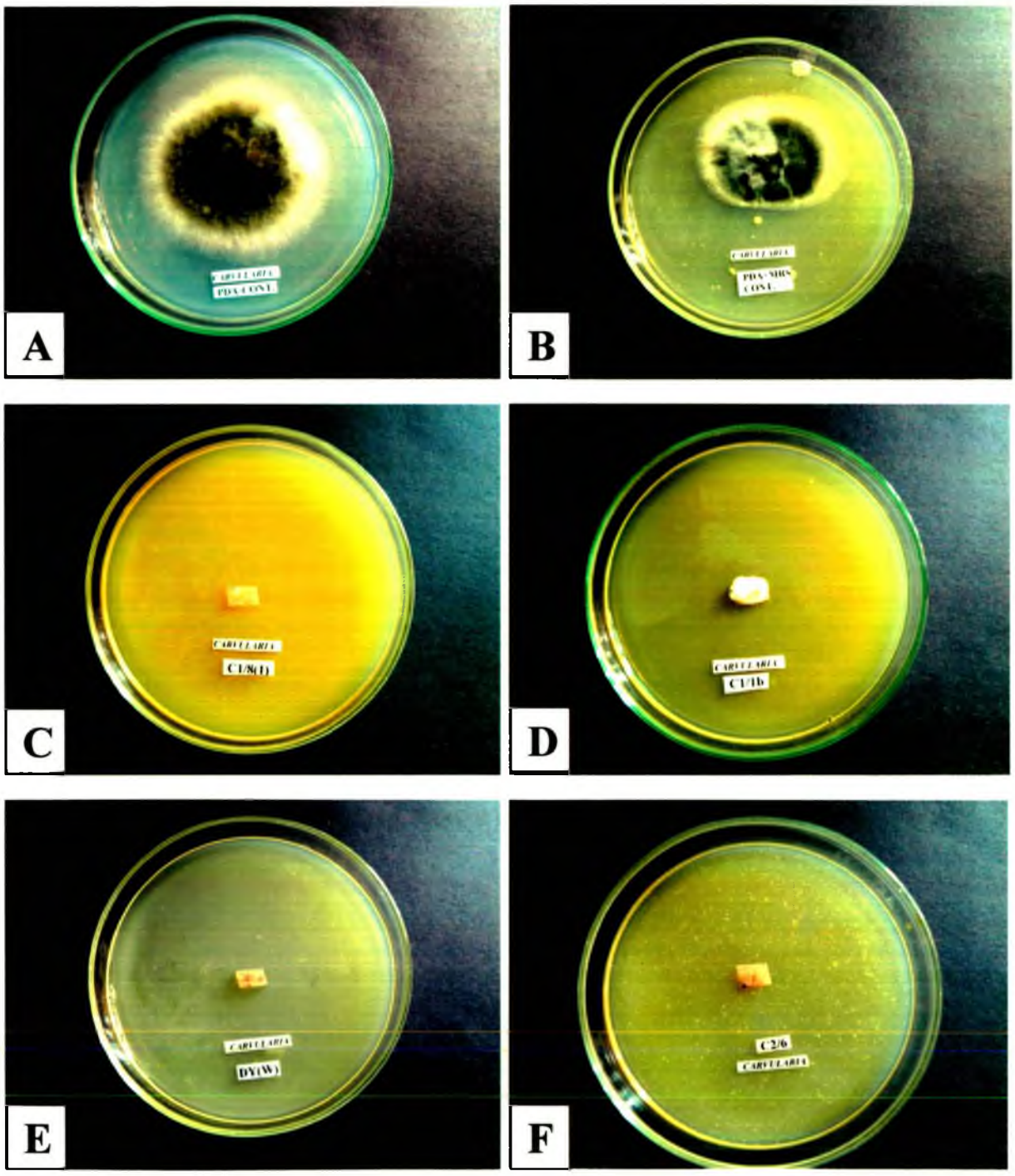

Fig. 37. Effect of culture filtrates of the selected organisms on the growth of *Carvularia lunata*. A and B showing normal growth (control) of *Carvularia lunata* on basal medium PDA and PDA with MRS broth. Probiotic isolates C1/8(1), C1/!b, DY(w) and C2/6 (C-F).

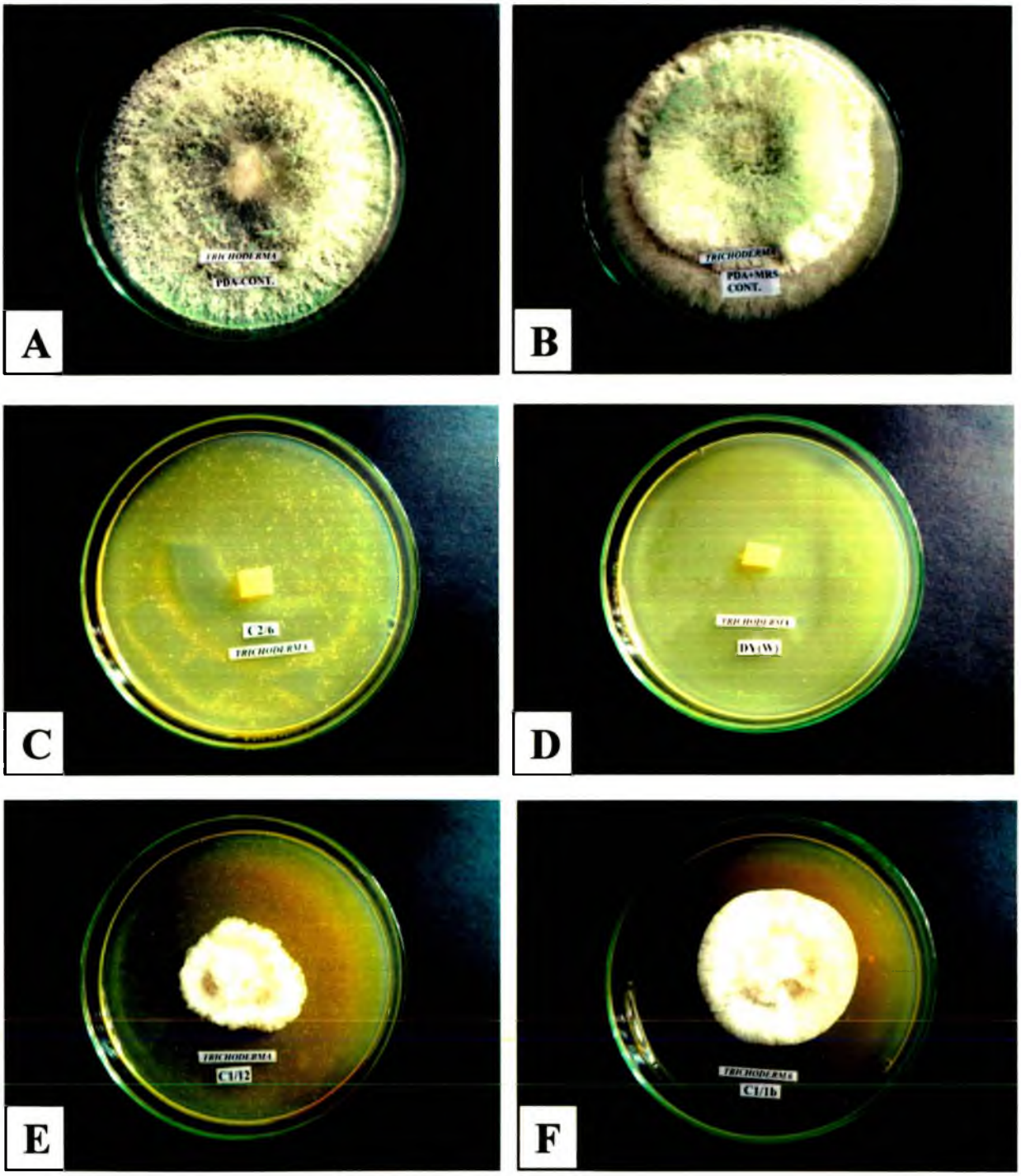

Fig. 38. Effect of culture filtrates of the selected organisms on the growth of *Trichoderma viride*. A and B showing normal growth (control) of *Trichoderma viride* on basal medium PDA and PDA with MRS broth. Probiotic isolates C2/6 , DY(w), C1/12 and C1/1b (C-F).

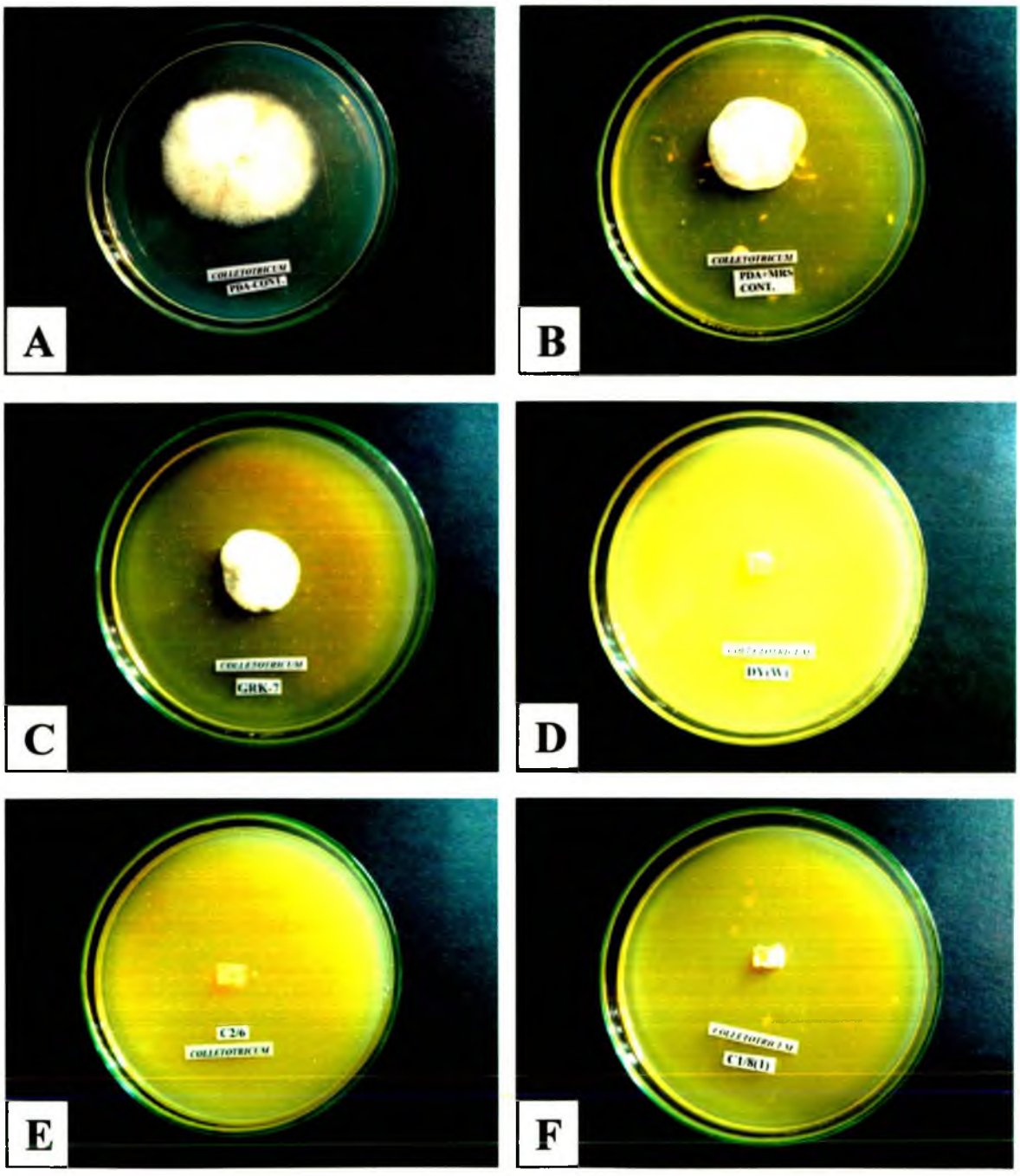

Fig. 39. Effect of culture filtrates of the selected organisms on the growth of *Colletotrichum coffeanum*. A and B showing normal growth (control) of of *Colletotrichum coffeanum*. on basal medium PDA and PDA with MRS broth. Probiotic isolates C2/6 (C), $DY_{(w)}$ (D), C1/12 (E) and C1/1b (F).

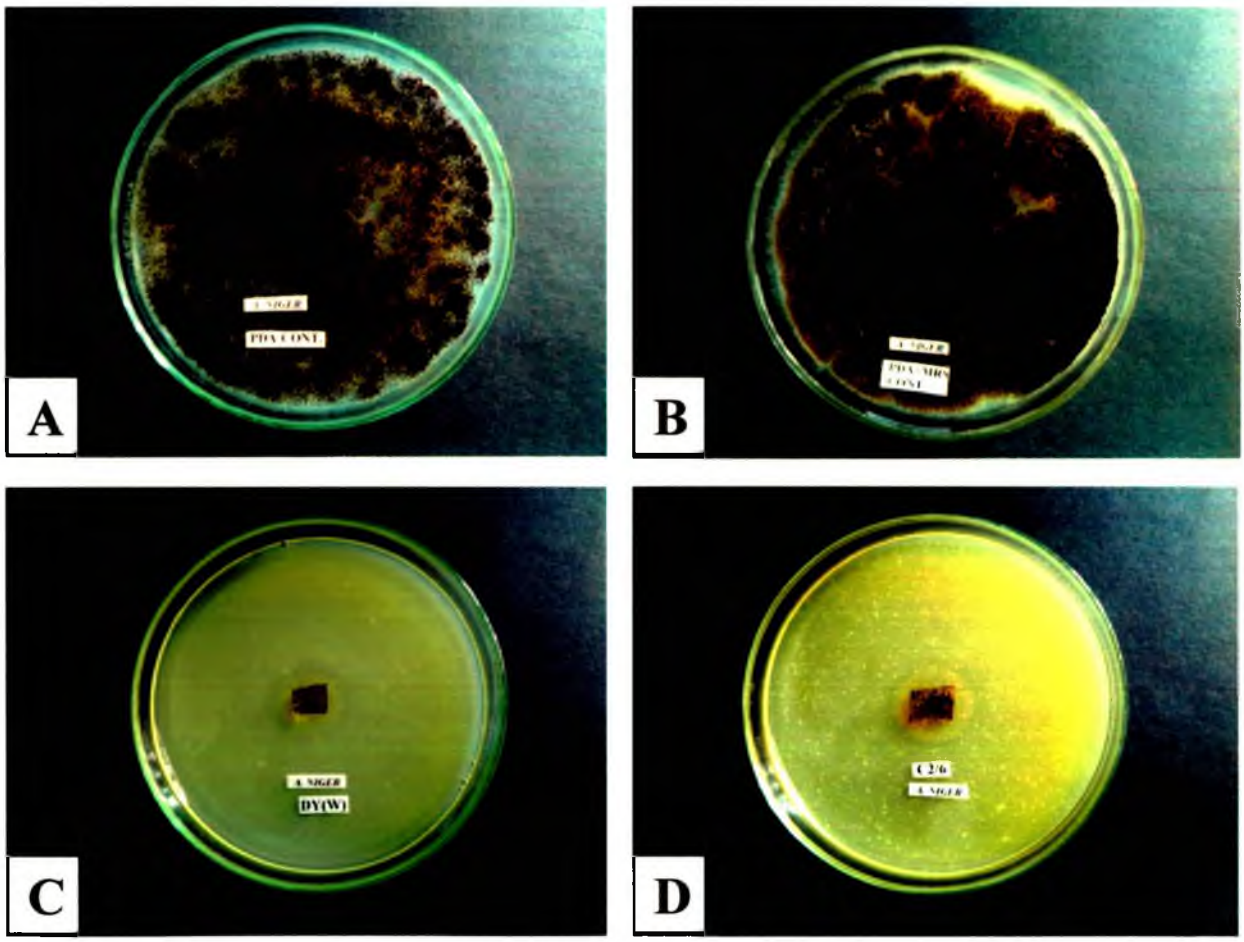

Fig. 40. Effect of culture filtrates of the selected organisms on the growth of *Aspergillus niger*. A and B showing normal growth (control) of *Aspergillus niger* on basal medium PDA and PDA with MRS broth. Probiotic isolates C2/6 (C) and DY(w) (D).

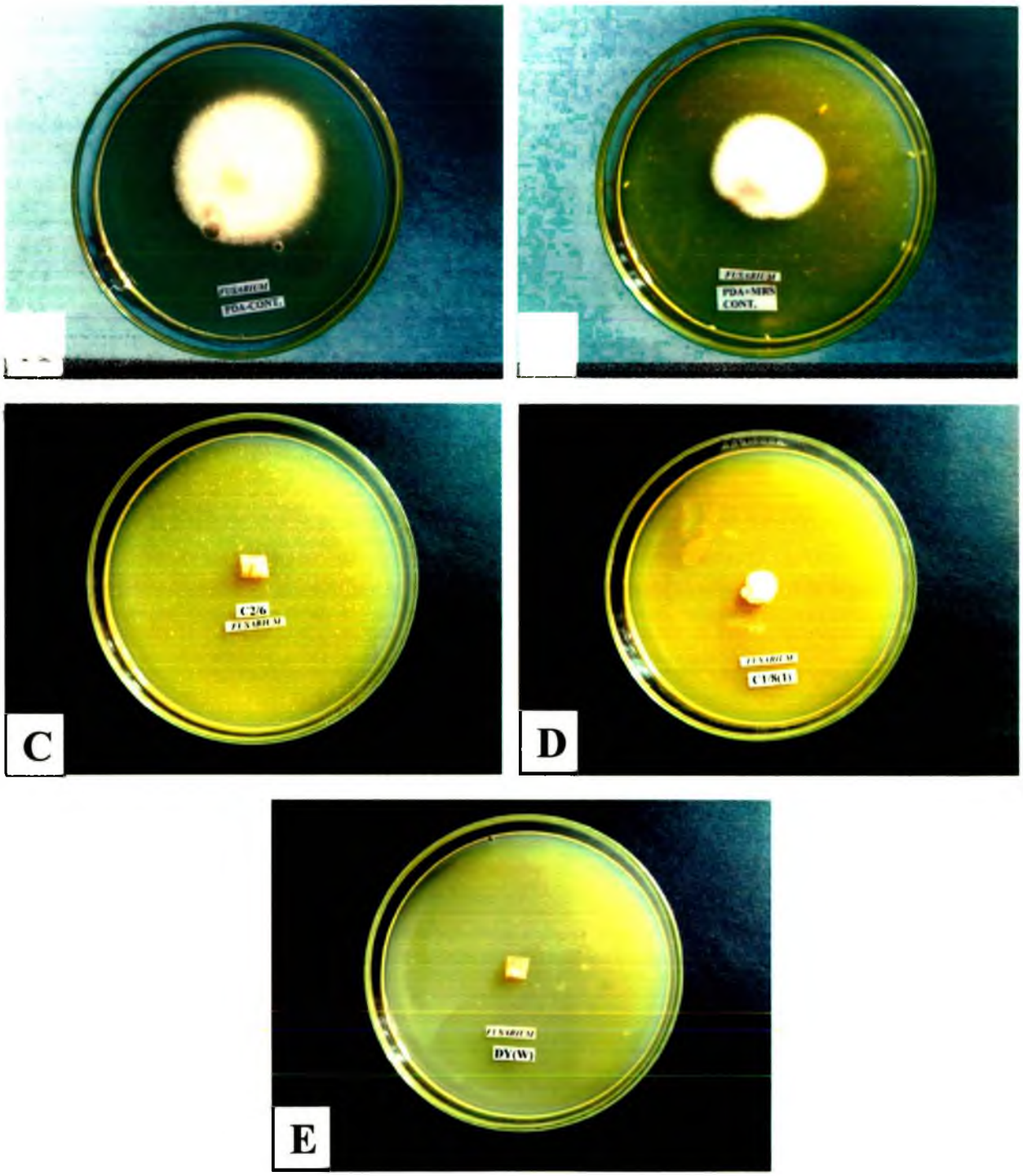

Fig. 41. Effect of culture filtrate of the selected organisms on the growth of *Fusarium moniliformae*. A and B showing normal growth (control) of of *Fusarium moniliformae* on basal medium PDA and PDA with MRS broth. Probiotic isolates C2/6 (C), C1/8(1) (D) and DY(w) (E).

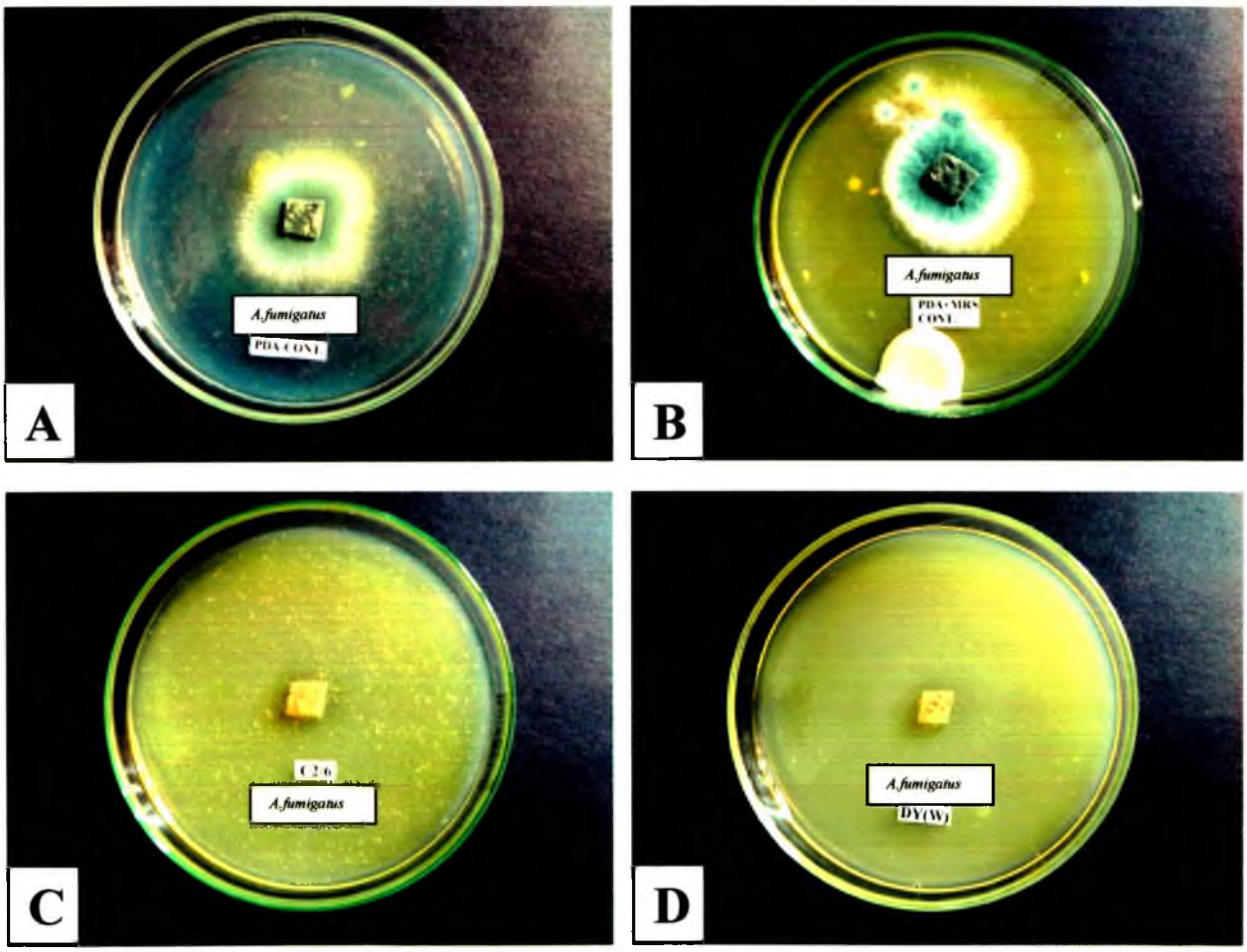

Fig. 42. Effect of culture filtrates of the selected organisms on the growth of *Aspergillus fumigatus*. A and B showing normal growth (control) of *Aspergillus fumigatus* on basal medium PDA and PDA with MRS broth. Probiotic isolates C2/6 (C) and DY(w)(D).

Nutritional potentiality of probiotic microorganisms

Over the last several thousand years, fermentation has been a major way of preserving foods. Microbial growth, either of natural or inoculated populations, causes chemical and textural changes to form a product that can be stored for extended periods. The fermentation process is also used to produce foods e.g. dairy foods, pickles, sauerkraut, gherkin etc. During the present research work, randomly selected probiotic isolates were used to ferment three food materials e.g. Cabbage, Soya bean and Star fruit juice. Different parameters of these food materials were measured before and after fermentation.

Preparation of food materials for fermentation:

Three samples were taken for fermentation:

Cabbage- Cabbage was cut into thin slices with the help of sharp knives and 50 gms were taken in a sterilized container. In this way six containers were prepared for further study.

Soya bean- 50 gm of dry soya bean sample was weighed and soaked in water for 24 hours. During this time, water was changed for several times to avoid unexpected contamination. Then, soaked soya bean was kept in sterilized container.

Star fruit juice- 800 g of Star fruit was mixed with 200 ml water and homogenized to make juice. For sterilization, the juice was passed through membrane filter (45µm pore size) and each container contained 50 ml juice for fermentation.

Cabbage and soya bean samples were autoclaved for 5 minutes at 121°C except one container of each sample which was used for natural fermentation.

Preparation of inoculum for fermentation:

During this research work, probiotic microorganisms were isolated both under aerobic and anaerobic conditions. For fermentation, both types of aerobic and anaerobic microorganisms were used. Randomly selected isolates were divided into three sets. Each set contained six microorganisms. Set 1 and Set 2 were anaerobic microbes and Set 3 was aerobic.

The organisms were cultured in modified MRS broth for 48hrs and from this fresh culture; 0.5ml inoculum was added into 50 ml medium and incubated at 30°C for 48hrs. To maintain anaerobic condition, cultures were kept in the anaerobic jar and paraffin and pyrogallic acid were used. Microbial cultures were centrifuged for 20 min at 12,000 rpm at 4°C temperature to form pellet and clear medium was decanted. Pellets were mixed with 10ml of 2.5% saline water and again centrifuged for 20 min to remove extra debris. Then the bacterial cells were separated from saline water and again mixed with 10 ml saline water (2.5%) and mixed with the substrates for fermentation.

Bacterial cells count:

Number of cells inoculated for fermentation was estimated by using two procedures, serial dilution (1:100) technique (Greenburg *et al.* 1980) and haemocytometer counting technique (Khaleque 1973) (Table 30).

Table 30: Numbers of cells of each microbe were recorded in the following table:

SET 1		SET 2		SET 3	
Isolate	No. of cells/ml	**Isolate**	No. of cells/ml	**Isolate**	No. of cells/ml
C1/7a	392500	**Cab2**	387500	**C1/3**	235630
C1/14	837500	**Cab3**	127500	**DJD1**	124400
C1/8(1)	200000	**Cab8**	240000	**CAB1**	317500
C1/10	362500	**GRK6**	137500	**CAB3**	230000
C1/4	165000	**C2/6**	118750	**DJD4**	147500
GRK5	152500	**DY(w)**	123750	**TBY5**	157500

Fermentation condition:

From each set of microbial suspension, 1 ml was added in the fermentation substrate. So 3 ml of saline water with microorganisms were supplied in the fermentation sample. All inoculated food samples were incubated at 30°C temperature for 14 days. In case of cabbage, one sample was kept for natural fermentation without sterilization. After 14 days fermented samples were used for further studies.

Fermented food samples analysis:

Determination of Moisture (water) content:

The estimation of moisture content of the samples was determined by following standard reference according to AOCA 950.46 (Official Methods of Analysis, 1997). About 10 gm of materials were weighted and allowed to sundry to remove sensible moisture. Then the samples were oven dried at 105°C to remove all moisture and cooled in desiccators. The processes of heating and cooling are repeated till a constant weight is achieved (Table 33).

$$\text{Moisture \%} = \frac{\text{initial weight- final weigh- final weight} \times 100}{\text{Weight of the sample}}$$

Nitrogen and Protein Determination (Kjeldahl Method):

Total protein content (crude protein, N× 6.25) was assessed by the Kjeldahl method according to AOAC 928.08 (Official Methods of Analysis, 1997).

Equipment:

- Digestion System K-438
- Auto Kjeldahl Unit K-370

Sample:

Cabbage, Soya bean and Star fruit juice

Procedure:

Digestion: the procedure for the complete digestion of organic material according to Kjeldahl is to oxidize the sample with concentrated sulfuric acid at high temperatures. Therefore, a digestion mixture of sulfuric acid, fermented samples and sulfate salts was used. The catalyst lowers the energy potential for oxidation process and the addition of the sulfate salts increased the boiling point of the sulfuric acid up to 370°C. This temperature remained constant during the digestion process. If the ratio of salts to acid is too high, the temperature of the digestion solution will rise up to 450°C (3 g catalyst/ml sulfuric acid conc.) because of the consumption of sulfuric acid for the oxidation. In consequence nitrogen losses (N2) will occur. The sample was cut into small cubes (maximum dimension: 2×2×2 cm) and frozen at -20°C. The frozen sample was homogenized thoughly after each grinding step. All cutter parts were chilled before preparation of each sample. Using an

analytical balance, 2 g of the homogenized sample was weighted on a nitrogen free paper; the sample including the paper was placed in a digestion tube. Add a spatula tip of stearic acid to prevent foaming during digestion and 2 Kjeldahl tablets (10g). About 20 ml of sulfuric acid (98%) were carefully added and the sample was suspended by gently swirling the tube. Additionally 2 blanks, only chemicals without sample, were prepared. The digestion system was preheated 40 min at the heating temperature 570°C. After 40 min the temperature is set at 400°C and the samples were digested for an additional time of 60 min. the samples were cooled down to ambient temperature prior to distillation (Table 31).

Table 31: Program on the Digestion system K-424 for digestion

Step	Heating temperature	Time (minute)
Preheating	570°C	40
Heating	400°C	60
Cooling	Room temperature	30

If the samples were not analyzed on the same day, dilute them with 50ml of water in order to prevent crystallization. Add water only to cooled samples, otherwise the reaction with the concentrated acid is violent and the sample may be lost. Gently swirl the tube to mix the digested sample with the water.

Distillation and titration:

If the samples have already been diluted with 50ml water, added only 20ml water for the distillation. The Autokjeldahl Unit was set according to the parameters listed in table 32.

Table 32: Parameter of the Autokjeldahl Unit K-370

Distillation		**Titration**	
Water	50 ml	Type	Boric acid
NaOH	90 ml	Titration	H_2SO_4. 0.25M
Reaction time	5s	Volume receiving sol.	60ml
Distillation time	240s	Min, titration-time	5s
Steam power	100%	Max- titration- vol.	40ml
Asp. Sample	Yes	Titration mode	Standard
Asp. Reac. Sol.	Yes	Titr. pH meas. Type	Endpoint
Stirrer speed	5	End-point pH	4.65
		Stirrer speed	7

Calculation:

The results are calculated as percentage of nitrogen. In order to calculate the protein content of the sample, the nitrogen content is multiplied with a sample-specific protein factor (6.25 for protein and 5.7 for feed sample). The following equations (1),(2) and (3) are used to calculate the results (Table 33).

$$w(N) = \frac{(Vsample - VBlank) \bullet z \bullet c \bullet f \bullet M \bullet (N)}{m_{sample} \bullet 1000} \rightarrow \quad .....(1)$$

$$\%\ N = w(N) \bullet 100\% \quad .....(2)$$

$$\%\ P = w(N) \bullet PF \bullet 100\% \quad .....(3)$$

W (N) = Weight fraction of nitrogen

V_{sample} = Amount of titrant for the sample (ml)

V_{Blank} = Mean amount of titrant for the blank (ml)

Z = Molar reaction factor (2 for H2SO4)

C = Concentration of the titrant, (mol/L)

F = Factor of the titrant (0.9920)

M (N) = Molecular weight of nitrogen (g/mol)

M sample = Sample weight (g)

1000 = Conversion factor

PF = Protein factor

%N = Percentage of nitrogen

%P = percentage of protein

Fat determination in the fermented food samples(Soxhlet method):

Lipids are soluble in organic solvents, but sparingly soluble or insoluble in water. The existing procedures for the extraction of lipids from source material usually involve selective solvent extraction and the starting material may be subjected to drying prior to extraction. Solubility of lipids is an important criterion for their extraction from source material and depends heavily on the type of lipid present, and the proportion of nonpolar (principally tri acyl glycerols) and polar lipids (mainly phospholipids and glycolipids) in the sample; therefore, several solvent systems might be considered, depending on the type of sample and its components (Table 33).

Samples used for fat:

Cabbage, Soya bean and Star fruit juice

Procedure:

I. Known amount of samples were taken and oven dried at 105°C and made powder with the help of mortar and pestle. Then powder samples were taken in conical flask.

II. 10 ml of petroleum ether was mixed with samples and kept in the dark chamber for 5 days for digestion.

III. The ether extract is filtered into a weighed conical flask. The flask containing the ether extract and powder sample mixture was washed 4 to 5 times with small quantities of ether and the washings were transferred for filtration.

IV. For the evaporation of petroleum ether filtrate, kept on the heat plate for 1min. then, flask with residue dried in an oven at 80-100°C and cooled in a desiccators and weighed.

$$Fat \text{ content (g/100g sample)} = \frac{\text{wt. of ether estract} \times 100}{\text{wt. of the sample (equivalent to fresh sample taken)}}$$

Estimation of total sugar in the fermented food samples (Ranganna 1991):

Invert sugar reduces the copper solution of red, insoluble, cuprous oxide. The sugar content in a food sample is estimated by determining the volume of the unknown sugar solution required to completely reduce a measured volume of Fehling's solution (Ranganna 1991).

Total sugar: An amount of 50 ml of the clarified solution was taken in the burette. Equal quantities of Fehling's solution (5 ml of A and 5 ml of B) were taken accurately with the help of pipettes and mixed the solution into a 500 ml conical flask and 190 ml of water was added. When the colour was turned into red then one drop of methylen blue was mixed and the solution became bluish red; then sample solution was added drop wise to make the colour tomato red and at the end point of titration, added one drop of methylen blue was disappeared (Table 33).

Calculation:

$$\% \text{ of total sugar} = \frac{\text{Fehling's factor} \times \text{vol. of Fehling's soln.} \times \text{vol. of sample soln.} \times 100}{\text{Titrate} \times \text{weight of sample}}$$

Table 33: Value of different nutritional parameter of fermented food samples:

Fermented Sample	Moisture content (%)	Protein content (%)	Nitrogen content (%)	Fat content (%)	Total sugar (%)
Cab control	94	0.351	0.053	4.70	2.16
Cab control Sterilized	93.7	0.376	0.060	5.05	2.23
Cab Fermented	94.6	0.376	0.060	1.60	0.78
Cab 1	94.1	0.348	0.056	1.34	0.73
Cab 2	93.38	0.354	0.057	2.66	0.76
Cab 3	93.95	0.435	0.053	0.89	0.96
Soya control	58.47	2.421	0.471	26.16	4.09
Soya sterilized	58.76	2.946	0.387	27.26	4.103
Soya 1	57.36	2.410	0.386	22.51	2.65
Soya 2	56.90	1.613	0.258	20.162	2.67
Soya 3	55.89	1.732	0.277	21.44	2.48
Star fruit control	ND	3.91mg/ml	0.63mg/ml	30.61	1.144
Star fruit 1	ND	3.33mg/ml	0.53mg/ml	28.39	-
Star fruit 2	ND	3.75mg/ml	0.55mg/ml	23.18	-

'-' Negative, 'ND' not done

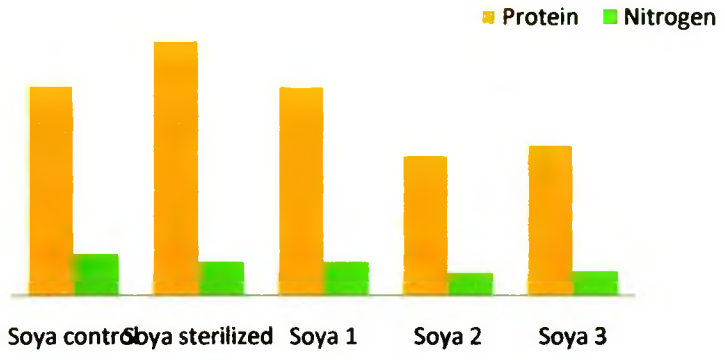

Fig 43. Estimation of Protein and Nitrogen of fermented Soya bean by isolated bacteria before and after fermentation

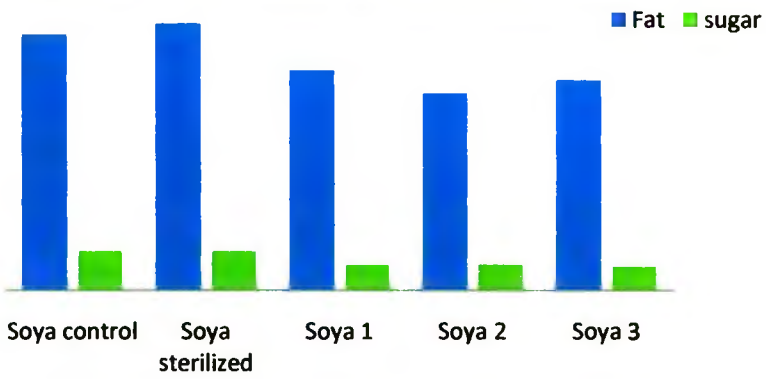

Fig 44. Estimation of fat and total sugar of fermented Soya bean by isolated bacteria before and after fermentation.

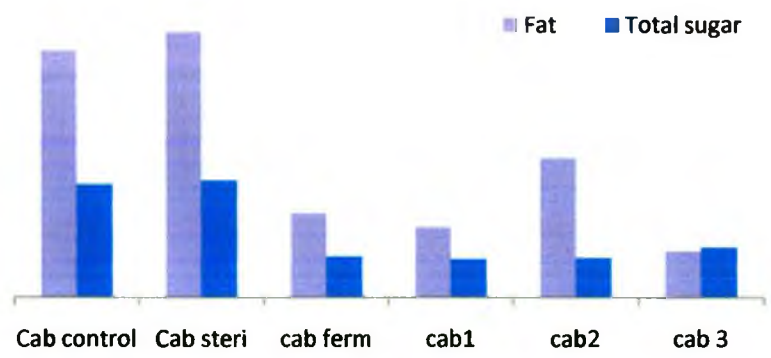

Fig 45. Estimation of fat and total sugar of fermented Cabbage by isolated bacteria before and after fermentation.

Discussion

Like any other organisms, man has also the natural instinct to eat, grow and survive. Eventually he has to die. But before that, he also tries to keep his progeny for the continuity of his own species. Termination of own life is sad but cruel reality. That is why even the most influential kings and conquerors tried every possible means to stretch their life and vitality for a little longer. Thousand and one methods and means were discovered, tried, told and retold. But still they die. During their life time, they use drugs and elixirs to keep themselves healthy and live for a little longer period.

Probiotics is only one such item, which is being used since the time of Abraham (Suvarna and Boby 2005). Previously people used probiotics without knowing any scientific explanation. With time, probiotics drew attention of scientists and now it has become a valuable commercial product.

During this research work, a wide range of fermented samples were used to isolate probiotic microorganisms such as fermented milk products (e.g. yogurt, cheese, fermented cream), vegetables (e.g. cabbage, cucumber) and date- palm juice.

Since probiotic microorganisms are nutritionally fastidious and many vitamins, amino acids, pruines and pyrimidines are needed to grow (Prescott *et al.* 2005), so special type of culture media were used to isolate and maintain their culture in *in vitro* condition e.g. MRS medium(Corry *et al.* 2003), Lee's medium (Atlas 1997), Bifidobacterial medium(Nebra and blanch 1999), Leuconostoc medium(Benkerroum *et. al* 1992) etc.

It is well established that probiotic organisms are microoaerophilic, aerotolerant and anaerobic in nature, so anaerobic condition was provided for isolation. After preliminary

screening 43 microrganisms were selected for detail studies. Among these, 13 organisms were isolated under aerobic and rest 30 under anaerobic environment.

Provisional identification was attempted by compairing all the available data with the standard description in the Bergey's Manual Vol.2 (Sneath *et al.* 1986).

Among the 43 selected organisms, 34 belonged to the genus *Lactobacillu*, 4 to *Leuconostoc*, 2 belong to *Streptococcus* and one was *Pediococcus*.

Two organisms remained unidentified. These two organisms C1/14 and Cab3, were isolated on bifidobacterial medium by following the method of Nebra and Blanch (1999). Isolated microbial colonies showed blue colour on bifidobacterial medium and colony diameter of C1/14 was 1.8mm and Cab3 2.0 mm, which were similar to Nebra and Blance's *Bifidobacteria*. However, there was a minor difference Bifdobacteria is obligate anaerobic, while C1/14 was aerotolerant and Cab3 was microaerophilic in nature.

Isolates DJJ4 and DJJ3 belonged to the genus *Strpetococcus*. These two isolates were facultative anaerobe, catalase negative and cells were arranged in chain or pair.

Isolate DJJ3 closely related to *S. parvulus* but differed in arginine hydrolysis, urease production and acid production from salicin, mannitol, sorbitol, trehalose and melibiose. Isolate DJJ4 was identified as *S. lactis* but showed dissimilarities in arginie hydrolysis, α hemolysis and acid production from raffinose.

Four isolates *viz.* DJJ1, FC2, DJD4 and TBY5 belonged to the genus *Leuconostoc*.

DJJ1 closely related to *Leuconostoc mesenteroides* subsp. *mesenteroides* but showed dissimilarities in the production of acid from melibiose and raffinose and production of acid clot and gas in litmus milk test.

FC2 resembled with *Leuconostoc mesenteroides* subsp. *cremoris* and showed difference in producing acid from fructose, sucrose, mannitol and mannose and hydrolysis of eculine. DJD4 was provisionally identified as *Leuconostoc paramesenteroides* and showed dissimilarities to proteolytic activity and production of acid from glucose, trehalose, maltose, melibiose and raffinose.

On the basis of the similarities, TBY5 closely resembled with the standard strain *Leuconostoc lactis* despite of its anomalies in the production of acid from glucose, trehalose, melibiose and raffinose and hydrolysis of esculine.

Only one isolates CAB1 was provisionally identified as *Pediococcus urinaeequi*. This isolate is catalase negative, facultative anaerobe, cells arranged in tetrads, some pairs, occasionally short chains may be seen but these are form by pairs of cells. It showed dissimilarities with the standard strain, in growth at 45°C and 50°C, pH 4.2 and 4.5 and acid production from rhamnose.

Probiotic isolates C3/4, C3/2 and C3/1b were closely related to heterofermnetative strain *Lactobacillus kefir*. These organisms showed dissimilarities in production of acid from melibiose, cellobiose, fructose, glucose and rhamnose and hydrolysis of esculin.

Isolate C1/3 closely resembled with homofermentative strain *Lactobacillus farciminis* and differed from this standard strain in production of acid from galactose, mannitol, mannose, salicin, sorbitol and rhamnose.

Isolate DJD1 was provisionally identified as *Lactobacillus vitulinus* which is a homofermentative strain and differed from the standard strain in production of acid from rhamnose, salicin, melibiose and mannitol.

Isolate CAB3 closely resembled with *Lactobacillus yamanashiensis*, showed dissimilarities in production of acid from mannitol, raffinose, salicin and sorbitol.

Isolate C1/7a resembled with facultative heterofermentative strains *Lactobacillus casei* subsp. *pseudoplantarum.* This isolate varied from strain *L. casei* subsp. *pseudoplantarum* in hydrolysis of eculin and arginine and production of acid from melibiose, raffinose and rhamnose.

Probiotic isolate C1/5a was closely allied to *Lactobacillus coryniformis* subsp. *torquens* and difference between them was the acid production from mannose and xylose.

Isolate no. C1/7 closely resembled with *Lactobacillus amaylophilus* and showed dissimilarities in the production of acid from lactose, mannitol, mannose, sucrose and xylose.

Isolate C1/12 was provisionally identified as homofermentative strains *Lactobacillus delbrueckii* subsp. *bulgaricus.* The considered isolate varied from the standard strain *Lactobacillus delbrueckii* subsp. *bulgaricus* in the production of acid from maltose, mannitol, sucrose, xylose and galactose and growth at 15°C.

Isolate C1/8(1) was closely allied to facultative heterofermentative *Lactobacillus casei* subsp.*pseudoplantarum* which was differed to this standard strain in production of acid from maltose, mannitol and sucrose.

Isolate no. C1/12a resembled with three standard strains- homofermentative *Lactobacillus jensenii*. The considered strain varied from the standard strain *Lactobacillus jensenii* in production of acid from lactose, mannose, salicin, sorbitol, xylose and rhamnose, hydrolysis of esculin and growth at 15°C.

Isolate C1/12s was closely related to two standard strains homofermentative *Lactobacillus farciminis*. The dissimilarities of the considered isolate with *Lactobacillus farciminis* in production of acid from cellobiose, mannitol, melibiose, trehalose, and xylose.

Probiotic isolate C1/10 closely resembled with standard strains- homofermentative *Lactobacillus crispatus*. C1/10 varied from standard strain *Lactobacillus crispatus* in several characteristics such as production of acid from cellobiose, mannitol, mannose, salicin and xylose and growth at 15°C.

Isolated organism C1/12b was provisionally identified as homofermentative strains- *Lactobacillus delbrueckii* subsp. *bulgaricus*. Difference between the considered strain and *Lactobacillus delbrueckii* subsp. *bulgaricus* - in the production of acid from galactose, maltose, mannitol, sucroase, xylose and growth at 15°C.

Isolate no. C1/16 resembled with facultative heterofermentative *Lactobacillus plantarum.* The difference between considered strain and *L. plantarum* - hydrolysis of esculin and arginine and acid production from xylose and rhamnose.

Isolated organism DY(w) closely related with obligatly heterofermentative strains- *Lactobacillus kefir*. The dissimilarities of the considered isolate with *Lactobacillus kefir* was production of acid from galactose, mannitol, sorbitol, sucrose and rhamnose.

Isolate DY(w)3 was closely allied to homofermentative strains *Lactobacillus sharpeae*. The dissimilarities of considered organism with *L. sharpeae* in production of acid from cellobiose, mannitol, manose, salicin, sucrose and xylose.

Isolated organism C1/2 resembled to obligately heterofermentative strain- *Lactobacillus bifermentan*. C1/2 showed dissimilarities with *Lactobacillus bifermentan* in production of acid from cellobiose, lactose, melibiose, salicin, sorbitol, sucrose and trehalose.

Isolate no. C2/2 was allied to facultative heterofermentative strain *Lactobacillus bavaricus*. C2/2 showed dissimilarities with *Lactobacillus bavaricus* in production of acid from mannose, salicin and rhamnose and hydrolysis of esculin and arginine.

Isolate C1/4 was provisionally identified as obligately heterofermentative strain *Lactobacillus hilgardii* and showed dissimilarities in several characteristics- hydrolysis of esculin and acid production from mannitol and xylose.

Isolate GRK5 resembled with facultative heterofermentative strains *Lactobacillus casei* subsp. *casei*. The standard strain *Lactobacillus casei* subsp. *casei* differed from the considered isolate in acid production from mannitol, mannose, salicin, trehalose and xylose.

Isolated organism GRK6 resembled with facultative heterofermentative strain *Lactobacillus coryniformis* subsp. *torquens*. The considered isolate showed dissimilarities with *Lactobacillus coryniformis* subsp. *torquens* in hydrolysis of esculin and arginine and acid production from xylose.

Probiotic isolate GRK7 was provisionally identified as *Lactobacillus salivarius* (homofermentative). GRK7 showed dissimilarities with the standard strain *Lactobacillus salivarius* in acid production from melibiose, raffinos, salicin, trehalose and xylose and growth at 15°C.

Isolate C1/8 was provisionally identified as facultative heterofermentative *Lactobacillus maltaromicus.* The standard strain *Lactobacillus maltaromicus* differed from isolate C1/8 in acid production from mannose and salicin.

C1/1 was closely related with facultative heterofermentative *L. plantarum.* C1/1 differed from *L. plantarum* in production of acid from cellobiose, mannose and trehalose.

C1/1b was closely allied to facultative heterofermentative strain *Lactobacillus casei* subsp. *casei. L. casei* subsp. *casei* showed dissimilarities with the considered strain in acid production from maltose, melibiose and raffmose.

Isolate C2/6 was provisionally identified as homofermentative strain *Lactobacillus salivarius. Lactobacillus salivarius* differed from the considered isolate in production of acid from salicin, rhamnose and trehalose, hydrolysis of arginine and growth at 15°C.

Isolate Cab1 was closely allied to facultative heterofermentative strain *L. casei* subsp. *rhamnosus*. Isolate Cab1 differed from *L. casei* subsp. *rhamnosus* in acid production from melibiose, raffmose, xylose and sorbitol.

On the basis of similarities, Cab2 was identified as homofermentative strains- *L. crispatus*. Cab2 showed dissimilarities with the standard strain *L. crispatus* in production of acid from mannitol, mannose, salicin and cellobiose and growth at 15°C.

Isolate Cab4 was provisionally identified as homofermentative *Lactobacillus sharpeae*. *L. sharpeae* showed dissimilarities in the production of acid from cellobiose, mannitol, mannose, salicin and sucrose.

Isolate Cab5 was closely allied to obligately heterofermentative strain *Lactobacillus collinoides* despite of its anomalies in production of acid from galactose, glucose, lactose, maltose, mannose, raffinose and sorbitol.

Isolate Cab6 was provisionally identified as homofermentative strain *L. crispatus*. The considered isolate differed from *L. crispatus* in production of acid from cellobiose, mannitol, mannose, raffinose and growth at 15°C.

Porbiotic isolate Cab8 resembled with homofermentative strain- *Lactobacillus farcimini*. The considered isolate showed dissimilarities with *Lactobacillus farcimini* in production of acid from cellobiose, mannitol, mannose, salicin, trehalose and xylose.

The majority of probiotic products available in marketplace contain species of Lactobacillus and Bifidobacterium, which are the main genera of Gram positive bacteria currently characterized as probiotics (FAO/WHO 2001).

Different species of probiotic microorganisms have been employed in the food industry, such as: *Lactobacillus acidophilus, L. casei, L. johnsonii, L. rhamnosus, L. thermophilus, L. reuteri, L. kefir, L. delbrueckii* subsp. *bulgaricus, Bifidobacterium bifidum, B. longum, B. brevis, B. infantis,* and *B. animalis* and *Streptococcus thermophilus* are found in a number of preparations such as traditional yogurts, frozen yogurts, and in desserts in some places (Knorr 1998; Senok 2009). Apart from *Enterococcus faecalis, E. faecium, Sporolactobacillus inulinus, Lactococcus lactis* subsp. *cremoris, Leuconostoc*

mesenteroides subsp. *dextranicum, Lactococcus lactis* subsp. *lactis, Saccharomyces boulardii, Pediococcus acidilactici, S. thermophilus,L. crispatus, L. fermentum* and *L. plantarum* are also considered as probiotic organisms (Tejada-Simon *et al.* 1999; Pestka *et al.* 2001; Holzapfel and Schillinger 2002).

During this research work, microorganisms were isolated from fermented food products as probiotic organisms belonged to different genus- *Lactobacillus, Streptococcus, Leuconostoc* and *Pediococcus* which are established as probiotic strains in industrial level. *Lactobacillus kefir, L. casei* subsp. *pseudoplantarum, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus jensenii, Lactobacillus crispatus, Lactobacillus plantarum, Lactobacillus casei* subsp. *casei, Lactobacillus casei* subsp. *rhamnosus, Leuconostoc mesenteroides* subsp. *mesenteroides* and *Leuconostoc mesenteroides* subsp. *cremoris* were found during the isolation period, which are well established as probiotic.

According to Hekmat and Reid (2006), two major characteristics of probiotic organisms- low pH tolerance and growth in presence of bile salt. Although various strains of lactic acid bacteria have been considered as probiotic, many are too sensitive to intense acidity and presence of bile salts in the human GI tract.

As bile salt tolerance is considered to be a major characteristic of probiotic organisms, these selected isolates were tested with different concentration of bile salts (0.5%, 1.0% and 2.0%). All the tested organisms grew well in up to 2% bile salt. Some of the isolates (e.g. C3/4, C3/2, C3/1b, DJJ1, DJD1, C1/5a, C2/2, C2/6, Cab1 and Cab3) grew even better in 2% bile salt medium.

Capability of growing at low pH is another characteristic of probiotic organisms, during this work the selected isolates were tested over a pH range of 3.5 to 7.5. All the isolates showed luxurious growth even at pH 3.5 and thus the organisms could be considered as probiotic.

The world health organization recently recommended to reduce the use of antibiotics both in human and animals and to increase efforts to prevent diseases by improving immunization coverage and reconsidering "older therapeutic approaches" such as bacterial interference. It is worth nothing that both aspects are part of the probiotic applications.

It is of general opinion that probiotic organisms influence positively on the host. The main points of consideration are help in digestion, enhance immunity, compete with and tribe out undesirable microorganisms from the digestive system of human and animals, blood cholesterol reduction, control of atopic eczema, prevention of colon cancer, infection of H. pylori, effects on viral infections and so on.

Antimicrobial activity is thought to be an important means for probiotic organisms to competitively exclude or inhibit invading bacteria (Carr *et al.* 2002; Roos and Holm 2002). These types of microbes do so by secreting non-specific antimicrobial substances, such as short chain fatty acids, hydrogen peroxide (Eschenbach *et al.* 1989), carbon dioxide, toxins with very narrow killing ranges such as bacteriocins, bacterocin-like inhibitory substances (Smith *et al.* 2007; Tagg and Dierksen 2003).

In the laboratory, the possible antimicrobial activity of the selected probiotic organisms were tested against nine pathogenic bacteria (*Staphylococcus aureus, Bordetella bronchiseptica, Escherichia coli, Micrococcus luteus, Staphylococcus epidermis,*

Enterobacter intermedium, Bacillus fastidious, Salmonella paratyphi) and six pathogenic fungi (Aspergillus niger, Aspeargillus flavus, Carvularia lunata, Fusarium moniliformae, Colletotrichum graminis and Trichoderma viride). Though the results were variable but all the tested organisms showed antimicrobial activity against all the pathogens, mentioned above.

Among the 43 isolates, C26 and Dy(w) showed strong antibacterial and antifungal activity. Isolates- Cab1, Cab2 and Cab5 inhibit growth of all test bacteria. Probiotic isolate C1/8(1) showed antagonistic activity against *Carvularia lunata, Fusarium moniliformae* and *Colletotrichum graminis*.

Probiotic organisms such as lactic acid bacteria, bifidobacteria, propionic bacteria have been used in the preservation of milk and vegetables for centuries. Today, a wide variety of strains are industrially used as starter cultures, co-cultures or bioprotective cultures to improve preservation, flavor and texture of milk, vegetable, meat and cereal products. The scientific basis of improved preservation is based on fermentation in which the pH value is lowered, the amount of available carbohydrates is reduced and many antimicrobial compounds are produced by fermenting bacteria. The ability to produce antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins implies an important biological role for bacteria and has been one of the key elements in the selection of these bacteria for probiotic use (Lee *et al*. 2009).

During this work cabbage, soya bean and star fruit juice were fermented by using randomly selected isolates and different parameters (e.g. moisture contents, protein and nitrogen contents, fat, total sugar) were estimated before and after fermentation.

In case of cabbage, there was no effect on protein and nitrogen contents after fermentation. But, fat percentage was reduced after fermentation. In the control samples, fat content was (5.05 – 4.70) % which was reduced to 1.34% (cab1), 2.66% (cab2) and 0.89 % (cab3). The amount of total sugar also reduced after fermentation.

After soya bean fermentation, protein and nitrogen percentage decreased from the control sample. Amount of fat and total sugar also reduced.

Total sugar content of fermented star fruit juice became nil. Its fat, protein and nitrogen content also reduced than control sample.

Now a days, considering the importance of probiotic organisms in the treatment and restoring our health, probiotics are being administrated in the form of capsules and this is quite popular among the physicians and patients. Even in Bangladesh a reputed pharmaceutical company has introduced probiotic capsules in the market. Many baby food manufacturers are already marketing large varieties of baby foods and supplements with prebiotic and probiotic factors.

Currently workers all over the world are paying more attention to the mysterious roles of probiotics in our health and longevity which remained unexplained for centuries. They are also trying to put their intelligence and innovation to retrieve in depth information for the successful exploitation of the benefits which probiotics has to offer. In problematic country like Bangladesh with millions of hungry and sick people, we must concentrate all our efforts and abilities for the welfare of our people Probiotics with their immense potentiality can definitely help us and our future generation.

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Appendix

Ammonium oxalate crystal violet solution (Claus 1995)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80 ml

Solution **A** and **B** were mixed.

Acidified $HgCl_2$ (SAB 1957)

$HgCl_2$	15.0 g
HCl (Conc.)	20.0 ml
Distilled water	100 ml

Arginine hydrolysis (Schaad 1988)

Peptone	1.0g
NaCl	5.0g
K_2HPO_4	0.3g
Phenol Red	0.01g
ArginineHCl	10.0g
Distilled water	1000 ml
Agar	3.0%
pH	7.2

Bifidobacterial medium (Nebra and Blanch 99)

Meat extract	2.0g
Yeast extract	7.0g
Starch	2.0g
L-cysteine hydrochloride	0.5g
Sodium chloride	5.0g
Peptone	5.0g
Tryptne	2.0g
Lactose	5.0g
Riboflavin	1 mg/L

Thiamine chloride hydrochloride	1 mg/L
Methylene blue	16 mg/L
Lithium chloride	2.0g
Propionic acid (99%)	5 ml/L
Distilled water	1000 ml
pH	5.5

Carbol-fuchsin solution (SAB 1957)

Solution-A

Basic fuchsin (90% dye content)	0.3 mg
Ethyl alcohol (95%)	10.0 ml

Solution-B

Phenol	5.0 g
Distilled water	95.0 ml

Solution **A** and **B** were mixed.

Caseinase test

Peptone	1%
Agar	1.5%

Skim Milk 0.5 ml in 15 ml medium

Congo red solution (SAB 1957)

Congo red (80% dye content)	2.0 g
Distilled water	100 ml

Deep glucose agar medium (SAB 1957)

Beef Extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g
Distilled water	1000 ml

Egg-yolk reaction medium (Sneath *et.al.* 1986)

Tryptone	10.0 g
Na_2HPO_4	5.0 g
K_2HPO_4	1.0 g
NaCl	2.0 g
$MgSO_4.7H_2O$	0.1 g
Glucose	2.0 g
Distilled water	1000 ml
1.5%egg-yolk was added aseptically	
pH	7.6

Esculine agar (Collins & Lyne 1984)

Esculine	1.0g
Yeast extract	3.0g
Ferric ammonium citrate	0.5g
Agar	7.5g
Distilled water	1000ml

H_2S production

Peptone Irone agar medium (Claus 1995)

Peptone	15.0 g
Proteose peptone	5.0 g
Ferric ammonium citrate	0.5 g
Sodium glycerophosphate	1.0 g
Sodium thiosulphate	0.08 g
Agar	15.0 g
pH	6.8
Distilled water	1000ml

Iodine solution (Claus 1995)

Iodine	1.0 g
Potassium Iodide	2.0 g
Distilled water	100 ml

Indole nitrate broth (Biolife 1999)

Peptone	20.0
Na_2HPO_4	2.0
Glucose	1.0
KNO_3	1.0
Agar	1.0
pH	6.8

Kovac's reagent (SAB 1957)

Para-dimethyl-amino-benzaldehyde	5.0 g
Butyl alchol	75 ml
HCl (Conc.)	25 ml

KOH-Creatine solution (Bryan 1950)

KOH	40.0 g
Creatine	0.3 g
Distilled water	100 ml

Leuconotoc medium (Benkerroum *et al.* 1992)	**%**
Glucose	1.0
Bacto Peptone	1.0
Yeast extract	0.5
Meat extract	0.5
Gelatin	0.25
Calcium lactate	0.5
Sorbic acid	0.05
Sodium azide	75 ppm
Sodium acetate	0.25
Tween 80	0.1
Tomato juice	15
Vancomycin (Incepta)	30 µg/ml
Cysteine hydrochloride (LOBA CHEMI)	20 µg/ml
Tetracycline (Incepta)	0.5 mg/ml (dissolved in ethanol)
Distilled water	1000 ml
pH	5.3-5.8

Autoclave for 15 minutes at 121°C

Lee's medium (Atlas 1997)

Pancreatic digest of casein	18.0g
Yeat extract	10.0g
Lactose	10.0g
Sucrose	5.0g
Calcium carbonate	3.0g
Potassium phosphate	0.5g
Distilled water	990 ml
Bromcresol Purple (0.2% solution)	10 ml
pH	7.0 ± 0.2

Lactose Mineral agar medium (ATCC)

Lactose	15.0 g
K_2HPO_4	5.0 g
NH_4Cl	2.0 g
NaCl	1.0 g
$MgSO_4.7H_2O$	0.1 g
Yeast extract	0.1 g
Agar	20 g
Distilled H_2O	1000 ml

Autoclave for 15 minutes

Litmus milk (Harrigan and McCance 1976)

Skim milk	1000 ml
Litmus	10 ml of 4% litmus suloution

Sterilize at 121oC for 5 min, followed by steaming for 30 min on each of the two following days.

MRS medium (Corry *et al.* 2003)

Tryptic digest of casein	10.0g
Beef extract	8.0g
Malt extract	4.0g
Glucose	20.0g
Tween 80	1.0g
Dipotassium hydrogen orthophosphate	2.0g

Magnesium Sulphate.$7H_2O$	0.2g
Manganese (II) Sulphate. $4H_2O$	0.05g
Ammonium Citrate	2.0g
Sodium Acetate. $3H_2O$	5.0g
Agar	15.0
Distilled water	1000 ml
pH	5.7

Modified MRS medium

Protease peptone	5.0g
Beef extract	10.0g
Yeast extract	5.0g
Dextrose	20.0g
Tween 80	1 ml
Tri Ammonium Citrate	2.0g
Sodium Acetate. $3H_2O$	5.0g
Magnesium Sulphate.$7H_2O$	0.2g
Manganese (II) Sulphate. $4H_2O$	0.05g
K_2HPO_4	2.0g
L-cysteine hydrochloride	1.0g
Tryptic digest of casein	10.0g
Sodium thioglycholate	0.2%
Tomato juice	15%
Distilled water	850 ml
pH	6.5

Autoclaved 15 minutes at 121°C

MRS medium for fermentation (de Man, Rogosa and Sharpe 1960)

Peptone	10.0g
Yeast extract	5.0g
Tween 80	1.0 ml
Di potassium hydrogen phosphate	2.0g
Sodium acetate	5.0g
Triammonium citrate	2.0g
Magnesium Sulphate.$7H_2O$	0.2g
Manganese Sulphate. $4H_2O$	0.05g
Distilled water	1000ml
pH	6.5
pH indicator- Bromocresol purple	0.004%

Mueller Hinton II agar (Atlas 1997)

Acid hydrolysate of casein	17.0g
Beef extract	2.0g
Starch agar	1.5g
Distilled water	1000 ml
pH	7.3 ± 0.1

Autoclaved for 15 minutes at 121°C

Mercurochrome solutino (SAB 1957)

Merecurochrome	0.5 g
Distilled water	100 ml

Methyl red solution (Bryan 1950)

Methyl red	0.1 g
Ethyl alcohol	300 ml
Distilled water	200 ml

5% aqueous solution of Malachite green (Manual of microbiological method)

Distilled water	100ml
Malachite green	5.0g

Nitrate reductase medium (SAB 1957)

Peptone	5.0 g
Beef extract	3.0 g
KNO_3	0.1%
Glucose	0.1%
Sodium thioglycholate	0.2%
NaCl	0.5 g
K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2 g
pH	7.0

Nitrate reductase test reagents:

Reagent A: Sulfanilic acid – acetic acid solution:

Sulfanilic acid	8.0 g
5N acetic acid	1000ml (1 part chemically pure Acetic acid 2.5 parts distilled water).

Sulfanilic acid was dissolved in acetic acid and stored in brown glass bottle.

Reagent B: Dimethyl-α-napthalamine solution:

Dimethyl-α-napthalamine	6.0 ml
5N acetic acid	1000 ml

Stored in brown glass bottle.

Reagent C: Zinc dusts

The tubes of nitrate broth in duplicates were inoculated with test organisms and then incubated at 37°C for 72 hrs. After incubation, 1 ml of reagent A was added to the incubated tube and shaken. Then 1 ml of reagent B was also added to each tube and shaken well.

Oxidase test reagent (Collins and Lyne 1984)

Tetramethyl-p-phenylene-diamine dihydro-chloride	1.0 g
Distilled water	100 ml

Phenylalanine agar medium (Sneath *et al.* 1986)

Yeast extracts	3.0 g
Di-phenylalanine	2.0 g
Na_2HPO_4	1.0 g
NaCl	5.0 g
Agar	12.0 g
Distilled water	1000 ml
pH	6.8

Potato Dextrose Agar (Atlas 1997)

Glucose	20.0 g
Potato infusion (300g potato)	1000 ml
pH	5.6

Autoclaved for 15 minutes at 121°C

Determination of Phenyl alanine (Sneath *et al.* 1986)

Yeast extract	3.0g
Phenyl alanine	2.0g
Na_2HPO_4	1.0g
NaCl	5.0g
Distilled water	1000ml
PH	7.3

Propionate medium (Sneath *et. al.*1986)

A. trace element solution

Ethylene diamine tetra acetate (EDTA)	0.5g
Ferrous sulfate	0.2g
Zinc sulfate	0.01g
Manganese Chloride	0.003g
Boric acid	0.03g
Cobalt Chloride	0.001g
Nickel Chloride	0.002g
Sodium Molybdate	0.003g

H_2O-100ml

B.

Sodium Propionate	2.0g
Di Potassium Hydrogen Phosphate	0.5g
Magnesium Sulfate	1.2g
Potassium Chloride	1.0g
Trace element solution	40.0ml
Distilled water	920ml
Phenol red (0.04% alcoholic solution)	20.0ml

pH-6.8

Starch agar

MRS medium	
Soluble starch	2%
pH	7.2

Safranin solution (SAB 1957)

Safranin	0.5 g
Distilled water	100 ml

Simmon's citrate agar medium (Claus1995)

Magnesium Sulfate	0.2 g
Mono ammonium di hydrogen phosphate	1.0 g
Di potassium hydrogen phosphate	1.0 g
Sodium Chloride	5.0 g
Sodium Citrate	2.0 g
Bromothymol blue	0.08 g
Agar	15 g
Distilled water	1000 ml
pH-6.8	

Tyrosine agar medium (Sneath *et al.* 1986)

L-tyrosine	0.5 g
Distilled water	10 ml
MRS agar	1000 ml
pH	6.8

Tyrosine was sterilized separately and was added to the medium before inoculation.

Tomato juice lactate agar medium (Harrigan and McCance 1976)

Pancreatic digest of casein	10.0g
Yeast extract	10.0g
Tomato juice	200 ml
Glucose	10.0g
K_2HPO_4	0.5g
KH_2PO_4	0.5g
$MgSO_4.\ 7H_2O$	0.2g
$FeSO_4.\ 7H_2O$	0.01g
$MnSO_4.7H_2O$	0.01g
NaCl	0.01g
Calcium Lactate	5.0g
Agar	9.0g
Distilled water	800 ml
pH	7.0

Autoclave for 15 minutes at 121oC

Reagents for total sugar estimation:

I. **Fehling solution (A):** 69.29 g of copper sulphite (CuSO4.2H2O) was dissolved in water dilute to 1000 ml if necessary filter trough no.4 whatman filter paper.

II. **Fehling solution (B):** 346 of Rochelle salt (Potassium sodium tartarate, $KNaC_4H_4O_6.\ 4H_2O$) was dissolved and 100g NaOH in water and made up to 1000 ml.

III. **Methylene blue indicator:** 1mg of methylene blue was dissolved in 100 ml of water.

IV. **Concentrated HCl acid**

V. **40% NaOH solution:** 40 g of NaOH dissolved in 100 ml distilled water and filtered the solution.

VI. **Standard sugar solution:** An accurately weighed amount of 1.009 g of Dextrose was taken into a 100 ml volumetric flask and then made up to mark with water.

Urease broth (Rustigen and Stuart 1941)

KH_2PO_4	9.0 g
Na_2HPO_4	9.5 g
Yeast extract	0.1 g
Phenol red	0.01 g
Distilled water	1000 ml
pH	6.8

Voges-Proskauar Broth (Bryan1950)

Peptone	5.0 g
Glucose	5.0 g
K_2HPO_4	5.0 g
H_2O	1000 ml
pH	7.2

PROBIOTICS AND HUMAN WELFARE