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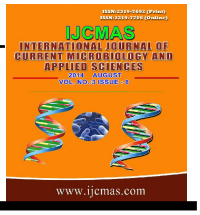

Original Research Article

Antibacterial activities of fermented whey on some selected Enteropathogenic bacteria

K.T.Adegbehingbe* and M.Bello

Department of Microbiology, AdekunleAjasin University, Nigeria
**Corresponding author*

A B S T R A C T

Whey sample was obtained and fermented for a period of four days. The pH, TTA, temperature and the proximate composition of the whey were monitored. The antibacterial activity of whey was assayed against *Escherichia coli, Pseudomonas aeruginosa, Streptococcus pyogenes, Proteus mirabilis, Salmonella typhii, Shigella sp., E. coli* (ATCC 25922), *Proteus mirabilis* (ATCC 25933), *Salmonella typhii* (ATCC 6539) and *S. flexneri* (ATCC 12022) using modified agar diffusion technique. Microbial counts increased throughout the fermentation period. Microorganisms isolated were *Lactobacillus plantarum, L. fermentum* and *Streptococcus lactis* dominated the whey while some pathogenic and toxigenic microorganisms such as *Pseudomonas aeruginosa, Staphylococcus aureus, Aspergillus flavus* and *Candida albicans* disappeared at the earlier stage of fermentation. The pH of the samples decreased while the total titratable acidity and the temperature increased throughout the fermentation period. Protein fat and ash contents of the fermented sample increased while the moisture and carbohydrate contents decreased. The whey had inhibitory effect on the growth of the entire test organisms. The zones of inhibition ranged from 12.00mm to 25.00mm in *Proteus mirabilis* and *Streptococcus pyogenes* respectively at 100%^{v}/v concentration. The highest MIC (70%^{v}/v) was observed in *Escherichia coli, Escherichia coli* (ATCC 25922) and *Streptococcus pyogenes* while *Shigella* sp had the lowest MIC (30%^{v}/v). However, minimum bactericidal concentration was not observed in any of the concentrations. The results of these findings suggested that fermented whey may be recommended for the treatment of infections caused by the tested organisms.

K e y w o r d s

Whey, fermentation, *Lactobacillus*, MIC, enteropathogens

Introduction

Antimicrobial agents are general nomenclature for all drugs or chemical substances that act on microorganisms either to kill or suppress their growth. An antimicrobial agent varies in their selective toxicity. Some act in a rather non-selective manner and have similar effects on all types of cells. Antimicrobial agents with selective toxicity are especially useful as chemotherapeutic agents in treating infectious diseases (Prescott, *et al*., 2008).

Antimicrobial agents can either be produced from microorganisms through their biochemical pathway or from plant extracts (Lappala, 1999).

Antimicrobial agents are known to show varying degree of action on microorganisms. They have been shown to have multiple target sites within thin microbial cells and the overall damage of these target sites results in microbecidal effect. The antimicrobial efficacy of any substance might give some information about the overall mode of action of such agent. The antimicrobial agent can exert their effect on microorganisms in any of the following ways; protein synthesis inhibition, inhibition of cell wall synthesis, inhibition of nucleic acid synthesis (Kimball and Jefferson, 2006; Prescott *et al*., 2008).

Whey is the yellowish liquid extract derived from production of fermented dairy foods. (Minekins *et al*., 1994). It is one of the components that can be separated from milk after curdling when rennet or an edible acidic substance is added. Whey is mainly water, but it also contains lactose, minerals, along with traces of fats and non-acid milk protein called "whey protein". Because whey contains lactose it should be avoided by those who are lactose intolerant. Whey has long been recognized as the best source of protein supplementation to repair tissue and to build muscle (Tunick, 2004; Krissansen, 2007). Whey and whey components are used by the food industry in a wide variety of applications on the basis of their excellent nutritional and functional properties (Foegeding *et al*., 2008; Tunick, 2008).

Bacterial causing diseases are self limiting; at times it may require antibiotic therapy (Prescott *et al*., 2008). However, because of the growing resistance of microorganisms to conventional antibiotics most of the commonly employed antibiotics are becoming ineffective. Whey contains a variety of factors and compounds which have been reported to have health promoting effects and prevent diseases (Lappala, 1999; Zemel, 2003; CDRF, 2006). Some of the factors are immunoglobulin, lactoferrin, lactoperoxidase, glycomacropeptide, bovine serum albumin, alpha-lactoglobulin and beta-lactoglobulin.

It is increasingly recognized as a functional food with a number of health benefits, having received growing interest as functional ingredients in dietary and health foods such as slimming foods, diets for the elderly and clinical foods. Bioactive whey ingredients such as bioactive proteins, which exert an additional health benefit for the consumer, are increasingly used in pharmaceuticals as well as nutraceuticals (Foegeding *et al*., 2002).

Whey protein is a mixture of globular proteins, its effects on humans health are of great interest and are currently being investigated as a way of reducing disease risk, as well as a possible supplementary treatment for several diseases (Krissansen, 2007).

The growing resistance of microorganisms to conventional antibiotics is becoming a serious concern to microbiologists and health care practitioner's all over the world. As a result, efforts are being made to develop antimicrobial agents from local sources for better chemotherapeutic effects but with less adverse effects (Oyeleke *et al*., 2008; Ismail *et al.,* 2011). The aim of this work was to determine the therapeutic properties of fermented whey against some pathogens of clinical importance.

Materials and Methods

Collection and preparation of whey

Fresh cow milk was obtained from the Fulani settlement in Akungba Akoko, Ondo State, Nigeria in a sterile container. The milk was briefly heated at 60^{o}C for 30 minutes, it was then allowed to cool and the whey was separated from the curd into sterile container using sterilized muslin cloth and fermented for 4 days.

Enumeration and isolation of microorganisms

Ten millilitres of the samples were homogenized with 90 ml sterile peptone water solution and further diluted as appropriate. The liquor was cultured on nutrient agar, Man de Rogosa and Sharpe (MRS) agar and MacConkey agar for the isolation of bacteria and potato dextrose agar for fungi using standard microbiological techniques. Bacterial cultures were incubated at temperatures ranging between 30 and 35^{o}C for 1–2 days while fungal plates were incubated at 25^{o}C for 2–5 days. MRS agar was incubated under anaerobic conditions simulated using a H_2/CO_2 generating kit (Oxoid) according to the manufacturer's instructions. Enumeration and isolation were done on daily basis during the fermentation period.

Pure bacterial isolates were then subjected to test such as Gram's reaction and biochemical tests and identified according to Holt *et al*. (1994) while fungi were identified according to Alexopoulous and Mims (1988).

Sterilization

The fermented whey was sterilized by filtering through Millipore membrane filter paper size 0.45µm and kept at 4^{o}C.

Collection of isolates

Clinical isolates of *Escherichia coli, Salmonella typhii, Shigella dysentarea, Proteus mirabilis Pseudomonas aeruginosa, Streptococcus pyogenes* were collected from Federal Medical Centre (FMC), Owo, Ondo State, Nigeria. While typed culture of *Escherichia coli* (ATCC 25922), *Proteus mirabilis* (ATCC 25933), *Salmonella typhii* (ATCC 6539) and *Shigella flexneri* (ATCC 12022) were collected from Federal Institute for Industrial Research Oshodi Lagos, Nigeria. The organisms were maintained on double strength Mueller-Hinton agar slants.

Standardization and inoculation of the organisms

All the organisms used were standardized to 0.5 McFarland standards. A 0.2ml aliquot of 24h old broth culture was dispensed in another sterilized 20ml Mueller-Hinton broth and incubated for 3-5 h. 1ml portion from the final broth is equal to 0.5 McFarland standard ($6x10^8$ cfu/ml) according to Oyeleke *et al.*, (2008). A sterile swab stick was dipped into the standardized broth culture and excess liquid was drained from the swab stick by pressing it gently to the inner side of the test tube containing the broth culture. The surface of the set media (Mueller-Hinton agar, Oxoid, England) was streaked with the swab stick.

Determination of antibacterial activity of the whey

Four wells of 5.00mm in diameter were then made in the solidified Mueller-Hinton agar plate seeded with the test bacteria using a sterile cork borer. Equal volume of whey was introduced into the wells made on the agar while distilled water and standard antibiotics (ciprofloxacin 20mg/ml) were used as the negative and positive control

respectively. The plates were incubated at 37°C for 24 hours and the diameter of zones of inhibition was measured.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the fermented whey

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the whey on the test bacteria were determined using agar diffusion method according to Prescott *et al*., (2008).

The proximate analysis and the determination of the physico-chemical parameters of the fermented whey

The moisture content, ash content, fat content, fiber content, crude protein and carbohydrate content of the fermented whey were determined and its total titratable acidity (TTA), pH and temperature of the whey were measured and recorded accordingly.

Statistical analysis

The data generated in the experiments were recorded and subjected to statistical analysis using standard procedure. The standard errors (SE) and critical differences (CD) at 5% level of significance

Results and Discussion

The microbial loads of the samples increased progressively as the fermentation progressed (Table 1). Total bacterial counts ranged from 7.37 X 10^3 cfu/ml to 9.67 X 10^8 cfu/ml while lactic acid bacterial counts increased from 5.02 X 10^3 cfu/ml to 5.02 X 10^3 cfu/ml. Fungal count was not as higher as total bacterial counts and lactic acid bacterial counts but ranged from 4.13 X 10^2 sfu/ml to 5.02 X 10^4 sfu/ml.

Five genera of bacteria and three genera of fungi were isolated and identified in the fermenting cheese whey (Table 3). They include *Streptococcus lactis*, *Lactobacillus plantarum, L. acidophilus, Pseudomonas aeruginosa, Bacillus* subtilis, *Saccharomyces cerevisiae* and *Aspergillus flavus. Lactobacillus plantarum* and *L. acidophilus* occurred throughout the fermentation period and were frequently isolated while *S. lactis* from day 2 till the end of the fermentation period. *Pseudomonas aeruginosa, B. subtilis*, *S. cerevisiae* and *Candida albicans* were only isolated up to the second day, while *S. aureus* and *A. flavus* were isolated in the first and the second days of the fermentation period respectively.

Table 3 shows the pH, total titratable acidity and the temperature of the fermenting whey. The pH of the sample was observed to decrease drastically as the fermentation progressed from 7.73 to 3.52 while the total titratable acidity increased from 4.2% to 24.63%. Temperature also increased and ranged from 27^{o}C to 33^{o}C.

The proximate analysis revealed that fermentation significantly affected the nutrient contents of the whey sample (Table 4). Increases were observed in the protein content (4.76-13.00)%, fat content (3.74-6.64)% and the ash content (2.76-5.45)% after fermentation. However, carbohydrate content decreased (9.06-4.69)% after fermentation while fibre content was not detected throughout the fermentation period. The fermented cheese whey used had inhibitory effects on the growth of all the test organisms used in this study (Table 5). Diameter of zones of inhibition ranged from 12.00±0.01 mm (*Streptococcus pyogenes*) to 25.00±0.03 mm (*Proteus mirabilis*). The minimum inhibitory concentrations (MICs)

for *Proteus mirabilis, Pseudomonas aeruginosa, Salmonella typhii, Proteus mirabilis* (ATCC 25933), *Salmonella typhii* (ATCC 6539) and *Shigella flexneri* (ATCC 12022) was 50%^{V}/V while *Escherichia coli, Escherichia coli* (ATCC 25922) and *Streptococcus pyogenes* is 70%^{V}/V and *Shigella flexneri* had the least minimum inhibitory concentration of 30% ^{V}/V (Table 6).

There was no bactericidal action of the fermented whey against any of the pathogens because there were growth at all the concentrations for all the bacteria tested (Table 7).

Microorganisms identified in the whey had been observed in some milk fermented products. Savadogo *et al*., (2004) isolated *Lactobacillus fermentum, Predicoccocus species, Leuconostoc mesenteroides* and *Lactobacillus* species from Burkinafaso fermented milk. The presence of *Pseudomonas* specie could be as a result of contamination from environment during processing.

This was in agreement with Sakhale *et al.* (2012) and Nout, (1994) who reported that various instruments and primitive techniques of processing foods are possible sources of contaminants. The predominance of lactic acid bacteria might lead to the decrease in the pH value of the sample due to lactic acid production and invariably increase in the total titratable acidity (TTA) with increase in fermentation period as this corroborates the works of Sakhale *et al.* (2012).

Lactic acid bacteria have an essential role in the fermentation of foods and beverages. The functional properties in lactic acid bacteria improve preservative effect and add flavor and taste to various fermented foods (Soomro *et al*., 2002; Adebolu and Ademulegun, 2006). The production of lactic acid by *Lactobacillus* present in the fermented whey could further enhance its nutritional value (Naik *et al.* 2009). Increases in the crude protein, fat and ash contents might be due to the proliferation of the fermenting organisms in form of single cell protein (Ismail *et al.,* 2011).

The fermented cheese whey used had inhibitory effect on all the bacteria used in this study. The inhibitory effects might be as a result of proteins such as lacto-peroxides and lactoferrin which were present in the whey (Axelsson *el al.,* 1998). This could also be due to the reduction in the Low pH of the fermented whey that resulted from lactic acid produced particularly by lactic acid bacteria which dominated the suspension.

This could create an environment that is not conducive for the growth of other microorganisms. Other factors responsible for inhibition might be due to the presence of microflora of milk such as *Lactobacillus* species which have the ability to produce antimicrobial substances such as bacteriocins. Gilliland and Speck (1977) and Warny *et al*.(1999) reported that *Lactobacillus* species exhibit growth inhibitory effects on various Gram positive and Gram negative bacteria through production of hydrogen peroxide, bacteriocins and organic acids such as lactic and acetic acids. These substances inhibit growth of pathogenic bacteria and also alter the ecological balance of enteric commensals (Adebolu and Ademulegun, 2006).

Table.1 Average colony counts of microorganisms isolated from fermented whey

Day	Total viable count (cfu/ml)	LAB count (cfu/ml)	Fungal count (sfu/ml)
1	7.37×10^3	5.02×10^3	4.13×10^2
2	2.44×10^4	6.24×10^4	9.23×10^2
3	8.51×10^6	2.43×10^6	6.78×10^3
4	9.67×10^8	5.02×10^3	5.02×10^4

Table.2 Occurrence of the bacteria isolates from fermented cheese whey

Microorganisms	Days of fermentation			
	1	2	3	4
Streptococcus lactis	-	+	+	+
Lactobacillus plantarum	+	+	+	+
Lactobacillus plantarum	+	+	+	+
Pseudomonas aeruginosa	+	+	–	–
Bacillus subtilis	+	+	–	–
Staphylococcus aureus	+	–	–	–
Saccharomyces cerevisiae	+	+	–	–
Aspergillus flavus	–	+	–	–
Candida albicans	+	+	–	–

Table.3 Description of Physico-Chemical of Fermenting Cheese Whey

DAYS	pH	TTA (%)	TEM P(^{O}C)
1	7.73	4.22	28.50
2	6.40	7.50	29.40
3	5.24	12.42	31.80
4	3.52	23.63	33.00

Table.4 Proximate Analysis of fermented and unfermented cheese whey

Parameters (%)	Unfermented whey	Fermented whey
Moisture content	79.67	69.23
Crude protein	4.76	13.00
Fat content	3.75	6.64
Ash content	2.76	6.45
Fiber content	00	00
Carbohydrate	9.06	4.69

Values are means of four replicates

Table.5 Antimicrobial susceptibility pattern of bacteria species to fermented cheese whey

	Zones of inhibition (mm)		
Test bacteria	**100%^{v}/v**	**Dw**	**Cpx (20mg/ml)**
Proteus mirabilis	**25.00±0.03 (83)**	**NI**	**30.00**
Escherichia coli	**22.00±0.02 (79)**	**NI**	**28.00**
Pseudomonas aeruginosa	**20.00±0.04 (69)**	**NI**	**29.00**
Streptococcus pyogenes	**12.00±0.01 (46)**	**NI**	**26.00**
Salmonella typhii	**18.00±0.02 (67)**	**NI**	**27.00**
Shigella flexneri	**21.00±0.02 (78)**	**NI**	**27.00**
Escherichia coli **(ATCC 25922)**	**20.00±0.01 (71)**	**NI**	**28.00**
Proteus mirabilis **(ATCC 25933)**	**23.00±0.03 (77)**	**NI**	**30.00**
Salmonella typhii **(ATCC 6539)**	**20.00±0.02 (77)**	**NI**	**26.00**
Shigella flexneri **(ATCC 12022)**	**19.00±0.01 (68)**	**NI**	**28.00**

Values are means of four replicates ± Standard error, Percentage inhibition over control in parenthesis

NI = No inhibition, DW= Distilled water, Cpx= Ciprofloxacin

Table.6 Determination of minimum inhibitory concentration (MIC) of fermented whey on some selected bacteria species.

Organisms	Zones of inhibition (mm)					
	100%^{v}/v	**70%^{v}/v**	**50%^{v}/v**	**30%^{v}/v**	**Dw**	**Cpx (20mg/ml)**
Proteus mirabilis	25.00±0.03 (83)	15.00±0.02(50)	6.00±0.02(20)	NI	NI	30.00
Escherichia coli	22.00±0.02 (79)	14.00±0.01(50)	NI	NI	NI	28.00
Pseudomonas aeruginosa	20.00±0.04 (69)	12.00±0.03(41)	5.00±0.01(17)	NI	NI	29.00
Streptococcus pyogenes	12.00±0.01 (46)	8.00±0.03(31)	NI	NI	NI	26.00
Salmonella typhii	18.00±0.02 (67)	10.00±0.02(37)	5.00±0.02(19)	NI	NI	27.00
Shigella flexneri	21.00±0.02 (78)	16.00±0.02(59)	8.00±0.02(30)	3.00±0.01(11)	NI	27.00
Escherichia coli (ATCC 25922)	20.00±0.01 (71)	10.00±0.01(36)	NI	NI	NI	28.00
Proteus mirabilis (ATCC 25933)	23.00±0.03 (77)	9.00±0.02(30)	3.00±0.01(10)	NI	NI	30.00
Salmonella typhii (ATCC 6539)	20.00±0.02 (77)	14.00±0.03(54)	4.00±0.01(15)	NI	NI	26.00
Shigella flexneri (ATCC 12022)	19.00±0.01 (68)	13.00±0.02(46)	3.00±0.02(11)	NI	NI	28.00

Values are means of four replicates ± Standard error, Percentage inhibition over control in parenthesis

NI = No inhibition.

Table.7 Minimum bactericidal concentration (MBC) of the extracts on susceptible organisms

Test organisms	Minimum bactericidal concentrations (MBC) (mg/ml)			
	100%^{v}/v	70%^{v}/v	50%^{v}/v	30%^{v}/v
Proteus mirabilis	x	x	x	
Escherichia coli	x	x		
Pseudomonas aeruginosa	x	x	x	
Streptococcus pyogenes	x	x		
Salmonella typhii	x	x	x	
Shigella flexneri	x	x	x	x
Escherichia coli (ATCC 25922)	x	x		
Proteus mirabilis (ATCC 25933)	x	x	x	
Salmonella typhii (ATCC 6539)	x	x	x	
Shigella flexneri (ATCC 12022)	x	x	x	

X = No inhibition

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