

Microbiological Analysis of Milk Shakes in Peshawar City, Pakistan

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ABSTRACT

The purpose of this study was to analyze microbial life and to address safety issues of milk shakes locally available in road side shops and carts of Peshawar city, Pakistan. Total of 144 samples of different flavors were examined for the presence of microbial content as total bacterial load, total coliform count, fecal coliform count and total staphylococcal count. Majority of samples have higher bacterial load (10^2 - 10^9 cfu/ml) and total coliform count (10^3 - 10^6 cfu/ml). *Escherichia Coli*, *Citrobacter*, *Klebsiella* and *Salmonella* species were detected using different biochemical tests. 26 samples revealed the presence of fecal coliform contamination (10^4 cfu/ml) while 120 samples harbored staphylococci (10^6 cfu/ml). High percentage of drug resistance among biochemical isolates was found against commonly used antibiotics like Imipenem, Nalidixic acid, Piperaciline, Ceftriaxone, Vancomycine, Ampicillin, Amoxicilline, Ciprofloxacin, and Erythromycine. Furthermore, 66% *Escherichia Coli*, 60% *Klebsiella*, 51% *Citrobacter* and 25% *salmonella* were found to be ESBL positive. In general the analysis indicates unsatisfactory quality of milk shakes and need to be microbiologically controlled by regulatory authorities to ensure public health safety.

Keywords: Milk shakes, Microbial content, Antibiotic resistance.

INTRODUCTION

In developing countries, fruit and vegetable juices are considered as an affordable and readily available source of nutrition especially in summer. Such unpasteurized juices with attributes of fresh flavor are sold by road side shops and is prepared simply by mechanical extraction method. Final product in the form of juice is

consumed directly by the consumer without any treatment. Pathogenic microorganisms find their way into fruits and vegetables through damage surfaces that occur during the process of growth and harvesting. In addition equipment contamination, raw material contamination, improper handling, unhygienic conditions also facilitate the

entry of pathogens into juices of fruit and vegetables^{1,2}.

Several studies reported food borne disease associated with the consumption of these juices³⁻⁵. Factors that can act as a source of contamination includes the use of unhygienic water, unhygienic environment, swarming flies like fruit and house flies and airborne dust. It has been found that bacterial pathogen notably *E. coli*, *Staphylococcal Areus*, *Salmonella* and *Shigella Spp* are likely to be present in such juices⁶.

It has been found that water can act as major source of microbial contamination like coliform, fecal coliform and staphylococci species (*Spp*) in food juices preparation. In developing countries several laws and regulation are there to strictly maintain the quality of food juices but lack of implementation; manufacturer does not bother microbiological safety and hygiene issues. This result in high rate of disease transmission through these juices and other drinks and has become a serious health problem⁷.

In recent years especially in summer there has been increased rate of food borne illness in Peshawar, capital of Khyber Pakhtun Khwa, province of Pakistan. Different types of milk shakes are consumed daily by large proportion of the population. Majority of these shakes are available in road side shops or cart, busy market area and recreational places like parks, fun lands etc. It has been found in majority of cases that these places or shops are not fit from hygienic point of view. Moreover it is also noticed that most of the manufacturer uses locally available tap water for juices preparation, which can act as major source of bacterial contamination. Most of these milk shakes are prepare uses milk of open shops directly without any treatment which may act as an additional source of contamination. The personal hygiene of the

manufacturer and the servant who prepare these milk shakes is also not satisfactory as most them are illiterate and does not know anything about microbiological aspect of juices preparation. Furthermore we also came to know about the use of contaminated equipments which further enhance the transmission of bacterial pathogens. Keeping in view all these points, we conducted microbiological study of locally available different types of milk shakes to find whether these milk shakes are fit human health or not.

MATERIALS AND METHODS

Sample size and location

A total of 144 different types of milk shake samples were collected from selected areas of the city including Board Bazaar, Peshawar University, University Road, Peshawar Sadar and Peshawar city.

Types of milk shake samples

12 ml of strawberry (n=48), banana (n=48) and apple (n=48) milk shake samples were collected using standard method.

Samples collection and processing

All the samples were collected using sterile flasks and were processed shortly after collection. During processing, 10 ml of the sample was mixed with 90 ml of sterile water and then serially diluted up to 10⁵. Each dilution was distributed evenly using spread plate technique on nutrient agar plates and then incubated at 37°C for 24-48 hours. Following incubation, plates were examined for discrete bacterial colonies and numbers of colonies were counted using colony counter (Synbiosis 5108 Pegaus Court, USA). Colony forming unit per ml (cfu/ml) for each dilution was then calculated. Plates were then refrigerated at 4°C for further processing.

Total coliform count (TCC), fecal coliform count (FCC) and total staphylococcal count (TSC)

Standard microbiological techniques like TCC, FCC and TSC were done using MaConkay agar media, membrane fecal coliform (mFC) agar and mannitol salt agar medium (MSA) respectively utilizing spread plate technique. Bacterial load was determined by standard method as described by ICMSF, 1998⁸. The obtained results of the above tests were then compared with the limit of Gulf standard, recommended microbiological standards as described by Rehman *et al.*, 2011⁹.

Biochemical characterization

Standard biochemical tests namely lactose fermentation, citrate, triple sugar iron (TSI), indole, urease tests were done for identification of specific coliform member. Before biochemical characterization the preserved agar plate colonies were streaked aseptically on fresh agar plates for purification purpose and were then incubated at 37°C for 24 to 48 hours. District laboratory practice in tropical countries by Monica Cheesbrough., 2006 was used for coliform member's identification¹⁰.

Antimicrobial susceptibility test

Isolates of the biochemical tests were purified on fresh agar media and were then tested against routinely used antibiotics using kirby-bauer disk diffusion susceptibility test as described by Bauer *et al.*, 1966¹¹ on Mueller-Hinton Agar Media (CM337-Oxoid). Single colony of each isolate was then introduced into 2 ml of Mueller-Hinton broth and 0.5 McFarland standard was used for culture turbidity adjustment. Sterile cotton swabs were dipped into the suspension and then spread equally on the entire agar plates. Antimicrobial impregnated discs were then

placed on the surface of inoculated plates using multi-disc dispenser. Following incubation, results were noted according to developed criteria by NCCLS 1996.

ESBL Determination

ESBL (extended spectrum beta lactamase) activities of the biochemical isolates was determined using double disc synergy method as described by Jarlier *et al.*, 1998¹². Beta lactam antibiotic disc with potency of 30 µg like augmentin (AMC), cefotaxime (CTX), ceftazidime (CAZ) and azetronem (ATM) was used in order to determine ESBL of 10⁶ cfu/ml inocula. Disc of AMC was used as a central disc and CTX, CAZ, ATM were placed at a distance of 20 to 30mm from the central (AMC) and from each other on the surface of agar plate of testing isolate. Plates were then incubated at 37°C for 24 to 48 hours. Following incubation, zone of inhibition was observed around third generation cephalosporin and azetronem disc. Zone of inhibition around one or more cephalosporin discs that was extended to the side nearest to the co-amoxiclave disc, sign of ESBL positive producer organism.

RESULTS AND DISCUSSION

In most of the samples bacterial load was quite high than permitted by gulf standard. Highest bacterial load in strawberry, banana and apple samples was 5.1×10⁹, 9.3×10⁸ and 7.3×10⁹ consecutively while lowest bacterial load for the three types of sample was 2.9 ×10², 2.2×10² and 2.1×10². (Table.1)

Variable size, rod shape, pinkish color colonies were observed on MaConkey agar media representing gram negative coliform. Bluish and yellowish colonies were observed on mFC agar and mannitol salt agar indicating gram negatives, rod shape fecal coliform and bunch of gram

positive staphylococcus *Spp* respectively. Biochemical analysis revealed the presence of *E. coli* (n=85), *Citrobacter* (n=51), *Klebsiella* (n=88), and *Salmonella* (n=8) *Spp*. (Figure.1)

It is also noticed that most of the samples contain high coliform count than permitted and is not allowed by safe food consumption standards. Highest coliform count for strawberry, banana and apple samples was 2.9×10^6 , 3.8×10^6 and 4.9×10^5 respectively. Some of the samples also harbor fecal coliforms which are totally not permitted by gulf standards. It was also found that fecal coliforms were present in 26 samples out of 144 samples. Highest fecal coliform load for the three types of sample was 1.8×10^3 , 2.6×10^4 and 3.4×10^4 . (Table.1)

Furthermore it was also observed that some of the samples show high prevalence of coagulase positive staphylococcal *Spp*, responsible for different human diseases through production of toxins. High load of staphylococcal *Spp* is required for sufficient and effective toxin formation (10^5 - 10^6 per ml of the food)¹³. Staphylococcus *Spp* was found in 87 % of strawberry, 79% of banana and 90% of apple samples. High load of staphylococcal *Spp* for strawberry, banana and apple samples was 6.1×10^7 , 2.2×10^5 and 1.2×10^7 respectively. (Table.1)

Antimicrobial susceptibility test revealed the existence of highly resistance pathogenic bacteria in all three types of samples. It was found that *E. coli* was highly resistance against nalidixic acid (74%), ciprofloxacin (71%) and ceftriaxone (61%). *Klebsiella* was highly resistance against ceftriaxone (95%), ciprofloxacin (88%), ampicillin (78%), amoxicillin (76%), erythromycin (65%), piperaciline (60%) and nalidixic acid (57%). *Citrobacter* has been found to be resistant against amoxicillin (93%), erythromycin (92%) vancomycine (90%), nalidixic acid (89%), ampicilline

(88%), ceftriaxone (81%), piperaciline (78%). Similar resistance pattern was found for staphylococcal *Spp* against nalidixic acid (78%), piperaciline (81%), ceftriaxone (86%), vancomycine (92%), ampicilline (96%), amoxicilline (94%), ciprofloxacin (87%), and erythromycin (21%) (Table. 2)

Based on ESBL determination result, out of total 85 *E. Coli Spp* 56 was ESBL producer while the remaining was classified as ESBL negative. For *Klebsiella* 53 were found to be ESBL positive out of 88, while out of 51 *Citrobacter* isolates 26 were termed as ESBL producer. It was also found that 2 salmonella isolates were ESBL positive out of 8. (Figure. 2)

In the present study we found that most of the milk shakes throughout Peshawar city are not fit for consumption because most of the samples show high bacterial load, total coliform count (TCC), fecal coliform count (FCC) and total staphylococcal count (TSC).

In many samples we found that there is high bacterial load ranging from 2.9×10^2 to 5.1×10^9 for strawberry, 2.2×10^2 to 9.3×10^8 for banana and 3.4×10^4 to 4.9×10^5 cfu/ml for apple samples. Such high bacterial load is not permitted by gulf standard and thus makes these milk shakes unfit for consumption. Rashed *et al.* (2012) reported high bacterial load, ranged between 10^2 - 10^7 cfu/ml, in vendor fruit juices samples in Dhaka city, Bangladesh¹⁴. Bagde and Tumane. (2011) found most of the juice samples have high bacteria counts ranged between 2.0×10^6 to 1.0×10^5 cfu/ml in Nagpur, India¹⁵. Rahman *et al.* (2011) reported that majority of fresh juice samples contain total viable bacterial count up to 2.4×10^4 cfu/ml¹⁶.

We also found that most of the milk shake samples show high prevalence of coliform bacteria. Total coliform count of these samples was much higher than allowed by gulf standard. Total coliform count for

strawberry, banana and apple samples was 2.9×10^6 , 3.8×10^6 and 4.9×10^5 cfu/ml respectively. Ahmed *et al.* (2009) found that different types of vended squeezed fruit juices samples have *E. coli* ranging from 43 to $>2400/100$ ml in Dhaka city, Bangladesh¹⁷. Bagde and Tumane. (2011) observed that fruit juices were found to be highly contaminated by *E. coli* in India¹⁵. Rashed *et al.* (2012) reported high count of coliform (1.58×10^6 cfu/ml) was found in vended fruit juices in Dhaka city, Bangladesh¹⁴.

The presence of staphylococcal *Spp* was also found in the samples. Maximum load of staphylococcal *Spp* observed in our studies for strawberry, banana and apple milk shakes samples was 6.1×10^7 , 2.2×10^5 and 1.2×10^7 cfu/ml respectively. Tambekar *et al.* (2009) reported Staphylococci prevalence in fruit juice samples in fruit juices in India¹⁸. Ahmed *et al.* (2009) revealed the presence of staphylococci in squeezed fruit juice samples is also reported in Dhaka city¹⁷. Rashed *et al.* (2012) found staphylococci in 30 out of 41 samples and total staphylococcal count for vended fruit juices samples was 6.95×10^5 cfu/ml in Dhaka city, Bangladesh¹⁴.

Based on the result of antimicrobial susceptibility test, we came to know that most of the isolates of our study are resistance to commonly prescribed antibiotics. Because of this resistance by pathogens, treatment with these antibiotics is found to be unaffected and poses serious threat to public health. Rashed *et al.* (2012) also found high percentage of drug resistance against commonly prescribed antibiotics by pathogens present in vendor fruit juices in Dhaka city, Bangladesh¹⁴.

It is also noticed that isolates including *E. coli*, *Klebsiella*, *Salmonella* and *Citrobacter* also show extended spectrum beta lactamase activities based on ESBL determination test result. Lateef *et al.* (2004)

observed β -lactamase activity of ten different strains isolated from orange juices in Ogbomoso, Nigeria¹⁹. M.khalil-ur-rehman khan *et al.* (2001) observed β -lactamase positive *E. coli* and *staphylococcal Spp* isolated from food stuffs²⁰.

Keeping in view all the above mention points, preventive measures rather than curative measures should be adopted. Strict check and balance of the milk shakes quality in Peshawar city is required. Educating the manufacturers and servants of milk shakes shops about safety techniques and microbiological aspects of juice preparation can help in reducing these problems. The use of pure water and pasteurized milk for shakes preparation must be ensured. Personal hygiene and sanitization of the manufacturer and servants should be maintained. Sterile equipments for milk shakes preparation should be used. The shops should be located in less crowded areas. High resistance of the isolates is a sign of antibiotics misuse, which need to be addressed soundly to avoid any undesired situation. Further molecular analysis of the biochemical isolate should be conducted to understand them in a better way.

CONCLUSION

The present study is about microbiological analysis of milk shake locally available in Peshawar in order to ensure the safety of the public health. It has been found that majority of the milk shake samples have high percentage of bacterial load, total coliform count (TCC), fecal coliform count (FCC) and total staphylococcal count (TSC) which is much higher than the limits of gulf standard. High resistance of the biochemical isolates to commonly use antibiotics is another serious health concern. ESBL activities of *E. coli*, *Salmonella*, *Klebsiella* and *Citrobacter Spp* make things more worsen. In most situations

it was observed that manufacturer and servant who prepare these milk shake juices have no knowledge about the sources of contamination and safe food preparation techniques. Things like personal hygiene, environmental hygiene, use of pure water, clean utensils etc can help in reducing the rate of food borne illnesses. Proper check and balance and regularly monitoring the quality of these milk shakes by regulatory authorities must be ensured to avoid any undesired future food borne outbreaks.

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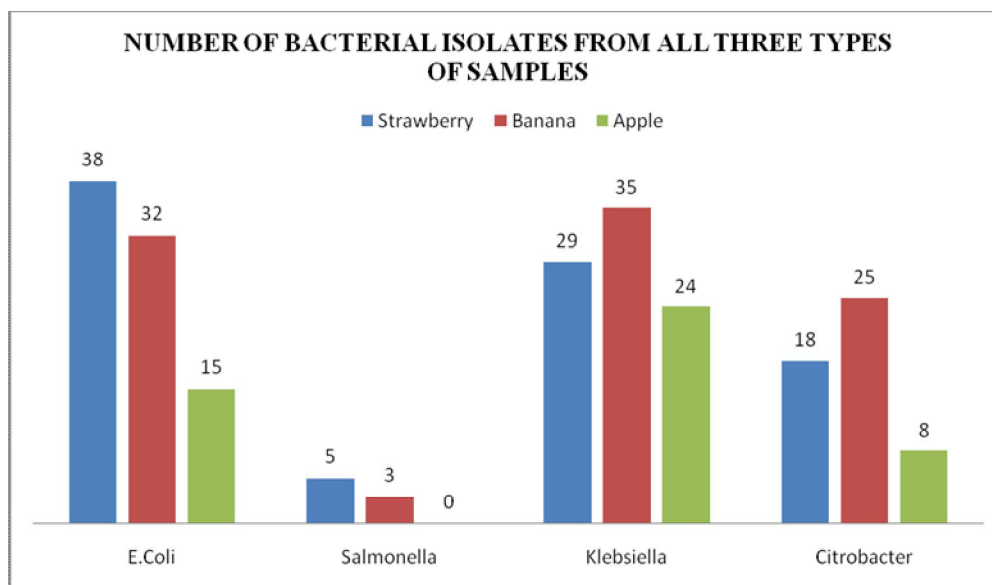


Figure 1. No of different bacterial isolates from milk shake samples

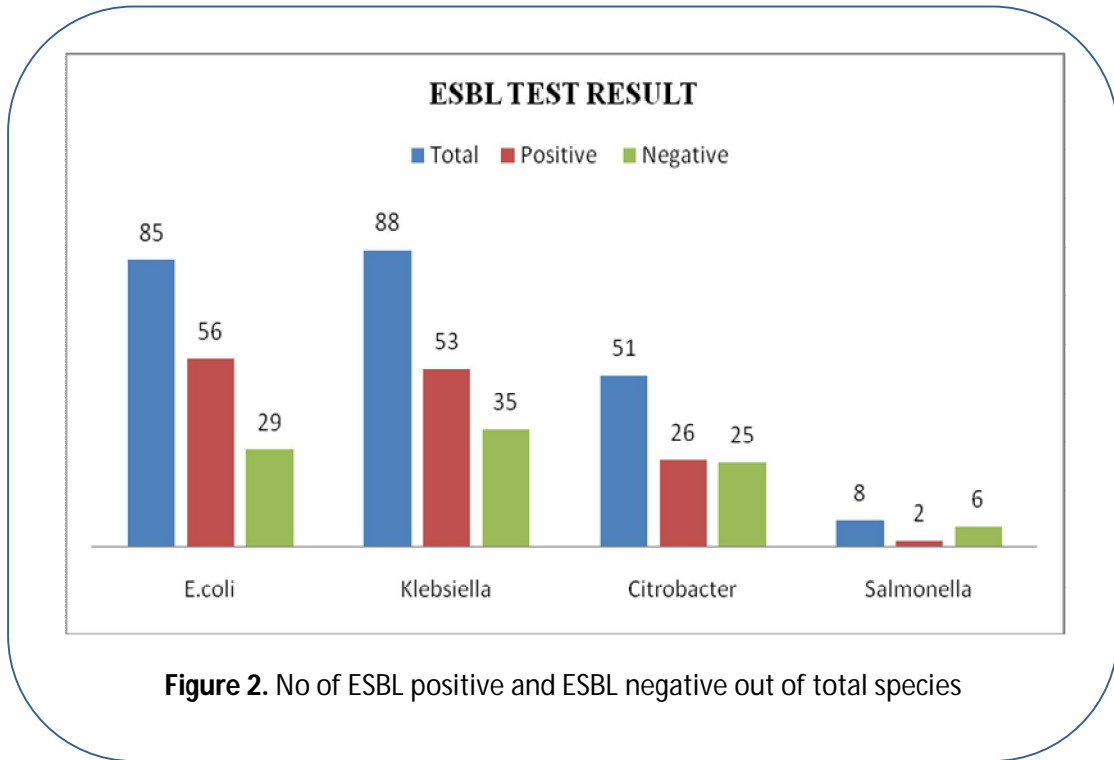


Table 1. TVC, TCC, FCC and TSC in Cfu/ml in different flavor milk shake samples

Sample No.	Sampling Area	Total Viable Count (TVC) Cfu/ml		Total Coliform Count (TCC) Cfu/ml		Fecal Coliform Count (FCC) Cfu/ml		Total Staphylococcal Count (TSC) Cfu/ml	
		Max	Min	Max	Min	Max	Min	Max	Min
S1-S10	B.B	7.3×10^8	8.2×10^4	5.2×10^5	2.1×10^2	7.3×10^2	0	3.2×10^5	1.9×10^2
B1-B10	B.B	1.5×10^8	6.9×10^2	1.3×10^5	1.7×10^2	1.2×10^2	0	1.3×10^4	1.1×10^2
A1-A10	B.B	3.1×10^7	1.9×10^4	2.6×10^3	1.2×10^2	3.7×10^2	0	2.1×10^4	1.6×10^2
S11-S10	P.U	2.1×10^9	2.9×10^2	2.9×10^6	1.1×10^2	2.7×10^2	0	6.1×10^7	1.9×10^2
B11-B20	P.U	4.5×10^7	2.2×10^2	1.5×10^5	1.2×10^2	2.2×10^3	0	1.9×10^2	1.2×10^2
A11-A20	P.U	2.3×10^8	3.9×10^4	1.9×10^5	1.1×10^2	3.6×10^3	0	3.7×10^5	1.1×10^2
S21-S30	U.R	5.1×10^9	2.8×10^3	2.2×10^6	1.7×10^2	1.8×10^3	0	3.5×10^4	1.6×10^2
B21-B30	U.R	2.9×10^9	4.5×10^4	3.8×10^6	1.1×10^2	2.6×10^4	0	2.2×10^5	1.6×10^2
A21-A30	U.R	2.7×10^8	3.2×10^4	1.0×10^4	1.1×10^2	2.7×10^2	0	3.7×10^5	1.8×10^2
S31-S40	P.S	9.1×10^8	2.1×10^3	2.9×10^6	1.1×10^2	3.6×10^2	0	3.3×10^6	2.2×10^2
B31-B40	P.S	1.4×10^9	2.1×10^3	2.4×10^4	1.3×10^2	1.1×10^4	0	3.3×10^3	2.3×10^2
A31-A40	P.S	2.3×10^8	2.1×10^2	2.2×10^4	1.9×10^2	2.1×10^2	0	3.2×10^4	2.6×10^2
S41-S48	P.C	3.6×10^9	2.4×10^3	2.4×10^6	1.2×10^2	1.3×10^2	0	3.1×10^4	1.1×10^2
B41-B48	P.C	9.3×10^8	6.4×10^2	2.5×10^5	1.4×10^2	1.8×10^3	0	4.2×10^3	1.7×10^2
A41-A48	P.C	7.3×10^9	2.6×10^4	4.9×10^5	2.1×10^2	3.4×10^4	0	4.1×10^4	1.8×10^2

Max=Maximum Min=Minimum B.B=Board Bazar P.U=Peshawar University
 U.R=University Road P.S=Peshawar Sadar P.C=Peshawar City

Table 2. Antimicrobial susceptibility pattern of different biochemical isolates in milk shakes samples (n=144)

Antibiotic	Symbol	Potency	Bacterial isolates									
			<i>E. coli Spp</i> n=85		<i>Klebiella Spp</i> n=88		<i>Salmonella typhi Spp</i> n=8		<i>Citrobacter Spp</i> n=51		<i>Staphylococs Spp</i> n=93	
			R	S	R	S	R	S	R	S	R	S
Imipenem	IPM	30 µg	2%	98%	3%	97%	1%	99%	2%	98%	5%	95%
Nalidixic acid	NALI	30 µg	74%	26%	57%	43%	15%	85%	89%	11%	78%	22%
Piperaciline	PIP	10 µg	36%	64%	60%	40%	18%	82%	78%	22%	81%	19%
Ceftriaxone	CEF	30 µg	61%	39%	95%	5%	21%	79%	81%	19%	86%	14%
Vancomycine	VAN	30 µg	39%	61%	49%	51%	15%	85%	90%	10%	92%	8%
Ampicillin	AMP	10 µg	23%	77%	78%	12%	30%	70%	88%	12%	96%	4%
Amoxicilline	AMO	10 µg	27%	73%	76%	24%	11%	79%	93%	7%	94%	6%
Ciprofloxacin	CIP	5 µg	71%	29%	88%	12%	8%	92%	83%	17%	87%	13%
Erythromycine	ERY	15 µg	45%	55%	65%	35%	6%	94%	92%	8%	21%	79%

Spp=species n=Number R=Resistance S=Susceptible