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FRUIT- BLEND YOGHURT WHEY - A POTENTIAL SOURCE FOR ANTIBACTERIAL DRUGS

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ABSTRACT

The use and overuse of antibiotics have led to antimicrobial resistance which poses a catastrophic threat in the modern era, in combating infectious diseases. A remedy is to develop new antimicrobial drugs. In this regard, fermented milk products can be focused as they are well known for its health benefits. Reverse Phase High Performance Chromatographic (RP-HPLC) studies of the whey of fermented milk and with fruit additives (banana, jackfruit, and pineapple) and their antibacterial property were evaluated. The whey extracts were tested against eleven pathogenic strains namely, *Staphylococcus albus*, *Staphylococcus aureus*, *Staphylococcus citrus*, *Shigella sonnie*, *Salmonella typhi*, *Salmonella paratyphi*, *Seratia marcenscens*, *Citrobacter freundi*, *Klebisella pneumonia*, *Escherichia coli* and *Pseudomonas aeroginosa*, by disc diffusion technique. The results revealed that fortified whey had a larger number of peptides and is excellent as antibacterial compounds and they exhibit antibacterial property against a wide range of pathogenic bacteria. They can, therefore, form potential sources for natural food –derived antibacterial drugs.

KEYWORDS: Fruit Blend Whey, Antibacterial Peptides, Antibacterial Drug, RP-HPLC

Several researchers have explored the functions of fermented milk. Much of the factors that contribute to the multitude of functions elucidated by fermented milk are mostly the effects of milk protein hydrolysates. Proteolysis during fermentation may lead to novel peptides having specific, desirable qualities. Milk peptides are considered to be one of the exceptional macromolecule having multifunctional attributes such as antimicrobial, antioxidative, antihypertensive, immunomodulatory, etc. Many of these bioactive peptides are encrypted within the primary sequence of the native protein and are released during proteolysis. In fermented milk, the bioactive peptides are due to the liberation of lactic acid bacteria and/or as a result of proteolysis of milk-proteins. Many of the antimicrobial peptides are derived from whey proteins. Though several antimicrobial peptides are identified and purified much still remains to be unravelled. Many new potential antimicrobial peptides from milk proteins are being discovered using bioinformatics tool.

The effect of the different substrate in addition to milk in fermentation by, lactic acid bacteria may change the growth and proteolytic pattern of fermentation, leading to liberation of a new kind or group of peptides. In view of consideration of the above fact, an evaluation of protein hydrolysates in whey by the fermentative process of milk alone and in combination with banana, jackfruit and pineapple pulps of the edible portions were carried to find their potential as food derived antibacterial agents.

MATERIALS AND METHODS

Preparation of Fermented Milk and its Blends

10% (w/v) skimmed milk was prepared by reconstituting the skim milk powder(Himedia).2%(v/v) of stock culture(Country Yoghurt culture mix, NDRI, Karnal, India) was inoculated and incubated for 14 h at room temperature. For preparing blends with fruits, 10% (w/v) fruits namely banana, jackfruit and pineapple, pulps of the ripe, uncooked, edible part was added to the cooled milk as prepared above and was inoculated by 2%(v/v) of stock culture(NDRI, Karnal) and was incubated for 14h. Whey was prepared by centrifuging fermented milk at 3000rpm for 20 min.

HPLC Analyses for Peptides in Plain and Fruit Blend Whey

Peptides were separated from plain and fruit blended fermented milk by precipitation of intact casein and large peptides by adding 5% (w/v) trichloroacetic acid (TCA). The filtrates were analysed by RP-HPLC (Shimadzu HPLC Model CR 72E, Shimadzu, Kyoto, Japan) using Shimpack CLC ODS C18, 5□m column packed with spherical silica particle of 100 Å pore size (250 □ 4.6 mm) fitted with guard column (Shimpack G ODS) of 4 mm id □ 1 cm length. The temperature of the column was maintained at 39°C. After the column was equilibrated with solvent A (0.1% TFA) at a flow rate of 1 ml/min, peptides were eluted by a solvent B gradient (60% acetonitrile in 0.1% TFA) as follows: 0 to 30 min, 0 to

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60% B, 30 to 35 min, 60 to 100% B and 35 to 42 min, 100 to 0% B. Eluted peptides were monitored at 220 nm.

Antibacterial Testing of plain and fruit blend whey peptides

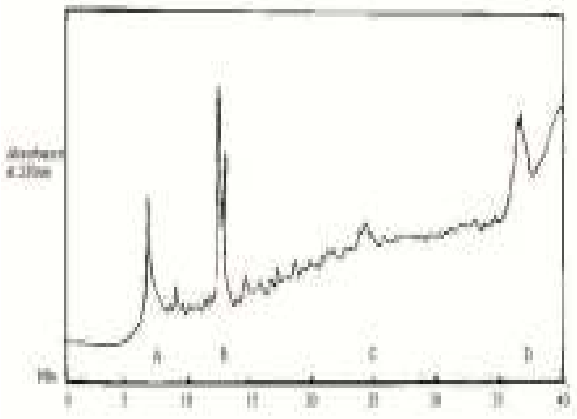
The whey from plain yoghurt whey and fruit blend whey were tested against eleven pathogenic strains namely *Staphylococcus albus*, *Staphylococcus aureus*, *Staphylococcus citrus*, *Shigella sonnie*, *Salmonella typhi*, *Salmonella paratyphi*, *Seratia marcenscens*,*Citrobacter freundi*, *Klebisella pneumonia*, *Escherichia coli* and *Pseudomonas aeroginosa.* For disc diffusion method commercially available blank sterile discs of 6 mm diameter were procured from Hi-Media Laboratories Pvt. Ltd., Bombay. 20 □l (0.02 ml) of acetone-methanol extract of whey from plain and fruit blend fermented milk, which was sterilised by passing through the microbial filter, was pipetted onto each disc. Disc diffusion method was carried out in Muller- Hinton agar medium and the zone of inhibition was expressed in millimeter.

OBSERVATIONS

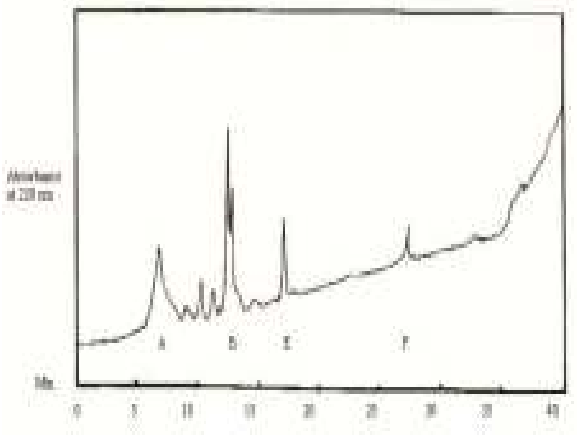
HPLC Profile of Peptides

As evident from the chromatograms Figures1a-1d, most of the peaks are eluted within the retention time of 20 min. It is noted that a major peak (Peak B) that eluted between 10 to 15 min shows a characteristic pattern with different treatments. This peak is present in all the samples analysed. Splitting of this peak is prominent in samples fermented with fruit additives, which might be due to very similar newly generated peptides. This may be interpreted as formation of entirely new species or a rather new fragment of protein with a slight difference in hydrophobicity, hence being eluted at different times. Thus it is proposed that with the addition of fruits there is change brought about in the cleaving pattern of milk proteins, the extent of cleavage being highest in pineapple combination.

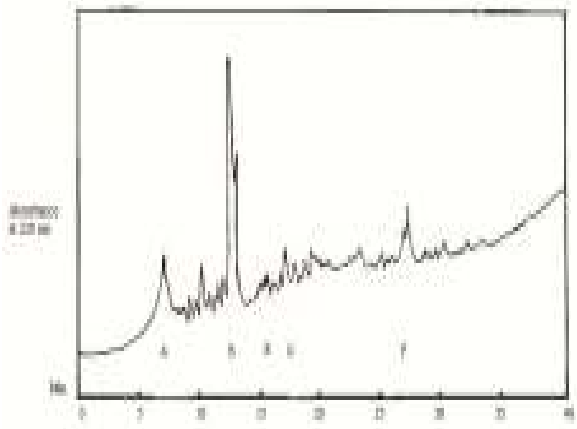
Characterisation and identifications of peaks have to be done and studied extensively to evaluate the exact nature and effect of fruits in the proteolytic system of yoghurt.



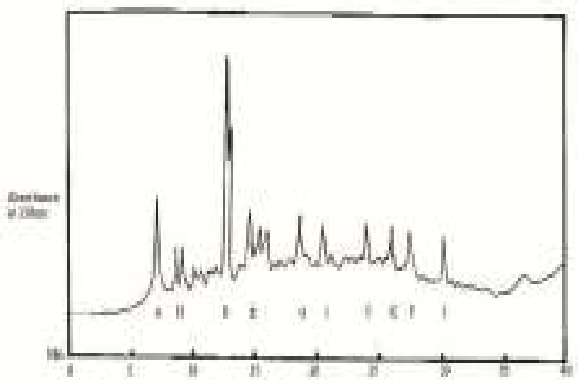
(1a)



(1b)



(1c)

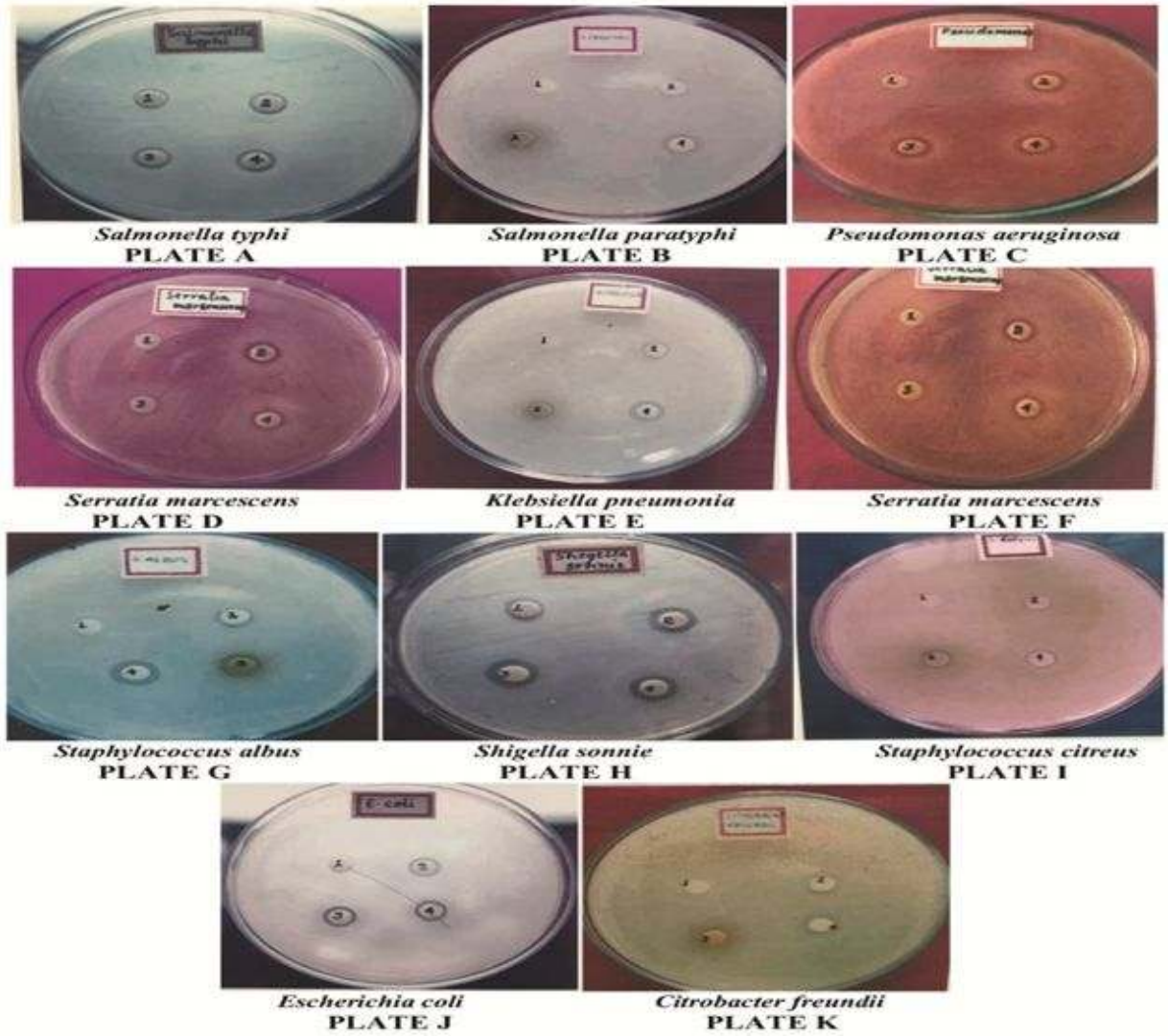


(1d)

Figure 1(a-d): RP- HPLC of plain, banana, jackfruit and pinapple yoghurt whey respectively

Antibacterial Property of Whey Peptides

As evident from the plates that whey obtained after fermenting milk with additives for 14 h has greater inhibitory activity against the test organisms. Of the various test, organisms screened those which showed inhibitory effects were *Staphylococcus albus, Staphylococcus aureus*, *Staphylococcus citreus, Shigella sonnie, Salmonella typhi, Salmonella paratyphi*, *Serratia marcenscens*, *Citrobacter freundii, Klebsiella pneumoniae, Escherichia coli,* and *Pseudomonas aeroginosa*.



1. Milk alone, 2. Milk+banana, 3. Milk+jackfruit, 4. Milk+pineapple

Figure 2(Plates A to K): Antibacterial activity shown by fermented milk alone and with fruit additives

In the present study, it was observed that milk initially fortified with fruit pulps and then kept for fermentation by lactic cultures showed higher antibacterial property than fermented milk alone. Addition of fruits may have further induced the generation of antibacterial substances by the lactic acid bacteria. Such substances are found to be peptides. Bacteriocins are intracellular and narrow spectrum antibacterials. The antibacterial substances concentrated from the whey of plain and fruit pulp yoghurts, which have been demonstrated to be peptides, might have derived predominantly from the milk proteins. Such a conclusion is based on the following- bacteriocins are the intracellular and narrow spectrum and the whey peptides are the extracellular and wide spectrum. Since one among the various factors of bacteriocidal and/or bacteriostatic action in lactic cultures is the presence of certain very low molecular weight peptides, it can be assumed that addition of fruit pulps to milk substrate may have changed the turnover of protein degradation into a much different way. Low pH, provided by the fruits, though adds to the antibacterial activity, by itself, but to a very lesser extent only. The methanol-acetone extracts of fruits alone showed no inhibitions against most gram-negative and negligible inhibition to gram-positive pathogenic strains under study (Plates not presented) which further proved that addition of fruits, while fermenting milk improved the quality of whey, by giving it pronounced antibacterial property than milk alone yoghurts through degradable peptides. The synergistic action of fruit additives in fermenting milk system is for certain, however, it has to be explored further to derive the active principle or principles for furthering their use. Further, it is observed that on storage beyond 15 days at 4 ^{o}C its activity is decreased. The possible reason may be the further degradation of the antibacterial substances (peptides).

Characterisation and identifications of peptides have to be done and studied extensively to evaluate the exact nature and effect of fruits in the proteolytic system of yoghurt. The vital role of such peptides in the enhanced antibacterial activity of fruit yoghurts could also be then ascertained.

DISCUSSION

From the present Reverse Phase High Performance Chromatographic (RP-HPLC) studies it is evident that more peptides are generated in fermented milk with additives. The pH, however, has an appreciable effect on the proteolytic activity of bacteria (El-Soda and Desmazeaud, 1982; Frey *et al.*, 1986). Addition of suitable fruit pulp to milk lowers the pH of fermented product considerably faster, which enhanced the release of peptides and amino acids.

Production of bacterial growth inhibitory peptides varies with respect to the duration of fermentation and contents of fermentative media. The fruit pulps added to milk for fermentation generates more of antibacterial peptides than milk alone. In the present study the generation of antibacterial peptides is in the following order- MP>MJ>MB>M. This implies that the bacterial growth inhibitory peptide varies with respect to the contents of fermentative media. Fruit blend yoghurts generate more of antibacterial peptides than plain yoghurt.

Misra and Kuila (1995) reported that addition of pineapple flavour 0.15% with 8% inoculum containing *Bacillus bifidum* and *Lactobacillus bulgaricus* incubated for 4 h at 45°C showed no antibacterial activity against the four test organisms namely *Escherchia coli*, *Shigella dysenteriae*, *Staphylococcus aureus* and *Bacillus cereus*.

Chopra and Prasad (1993) and Chopra and Gandhi (1989) have shown that whey plays an effective role in the production of inhibitory substances by the lactic cultures. Maria *et al.* (1992) showed that inhibitory effect of lactobacilli on the growth of *Shigella sonnie* was not due to pH alone but due to inhibitory substances as well, which are peptides.

CONCLUSION

Addition of fruits prior to fermentation lowers the pH which in turn enhances the proteolytic activity of lactobacillus releasing potent antibacterial peptides. The extract of these fruits alone shows no antibacterial activity revealing the role of lactobacillus in fermenting media in generating antibacterial peptides. The findings reported here makes the use of cheap and abundantly found fruits- banana, jackfruit and pineapple in the preparation of enzymatic digests of whey with antibacterial properties.

The present findings that addition of above mentioned fruits induced the generation of more antibacterial peptides in the fermenting milk can be utilized to manufacture value-added products like in formulation of new antibiotics. These natural compounds can turn to be the ingredients of future drugs to overcome the problem of treating infectious diseases where the pathogens show resistance to the existing antibiotics.

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