Characterization of Cellular Fatty Acids of *Listeria* Species and their Effect on Circulating Blood Monocytes

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Summary: *Listeria monocytogenes* NCTC 7973, *L. ivanovii SLCC* 2379 and *L. seeligeri* SLCC 3954 were found to contain 5 - 7.8 % (dry weight) chloroform- soluble lipids. All species exhibited, nearly similar fatty acid esters profile with little difference when grown at 37 °C. The study revealed the abundance of odd chain saturated fatty acids in all the three species of *Listeria.* Among all, in particular *ante-iso* are more prevalent than *iso-* forms. The high percentage of the C15 fatty acid ester was characteristic of each species but with some differences in the relative amounts were observed. C19 and C22 fatty acid esters were characteristic of *L. monocytogenes*. Whole cells of *L. monocytogenes* and *L. ivanovii* induced strong monocytosis in the infected animals (rabbits, mice, and rats) of varying degree of susceptibility. Similar effect was observed with crude lipid extract of *L.moncytogenes*. No such response was observed even when live *L. seeligri* cells or crude lipid of *L. ivanovii* were injected.

Introduction

There is a renewed interest in listeriosis in many countries due to the occurrence of food-borne outbreaks [1-3]. *Listeria monocytogene*s has been recognized as an important food borne pathogen of public health significance [4]. The Food and Drug Administration of the United States of America (US-FDA) has incorporated *Listeria* into the HACCP (Hazard Analysis Critical Control Point) concept for quality control in food industries [5]. Because of the intriguing nature of the bacterium, its metabolites, toxins (exotoxins), extracts/preparations and their biological activity have been the subject of much concern and discussion [6-10].

Infections with *L. monocytogene*s frequently can cause monocytosis in monogastric animals. A monocytosis comparable to that occurring during infections can be induced by a monocytosis producing agent (MPA) extractable from the bacterium [11]. There have been conflicting reports that MPA is present in greater amounts in virulent strains, or that MPA quantity is inversely proportional to the virulence of the organisms. Hence possible role of MPA in the pathogenesis of listeriosis is unclear [12].

The importance of listerial lipids and its relationship with MPA or to the virulence of *Listeria* is evident from the literature [13]. Previously studies have been focused on establishing the relationship between the chemical composition and biological activity of the lipids of *Listeria* species [14, 15].

Although the cellular fatty acids of *Listeria* species have been studied but no information is available about the relation between chemical composition of the lipid and the virulence of *Listeria*. [15]. The exact nature of the stimulus, due to lipids to the monocytes precursor is also not known. It may simulate the cells of mononuclear phagocytic series, increase the generation time of promonocyte in bone marrow and decreases the half time of circulating monocytes *in vivo* [16].

The present study was accordingly initiated to accumulate more information regarding the cellular fatty acid composition, their biological activity and their role in the pathogenicity of the bacterium. In addition, we also report the monocytosis producing activity of the whole cells and lipids of *Listeria* species.

Results and Discussion

Three different species of *Listeria* grown at 37 °C in TSB were used for the determination of lipids and their FAs composition. Of these species, highly pathogenic *L. monocytogenes* NCTC 7973 gave 77.8 mg of crude lipid g^{-1} dry weight, *L. ivanovii,* a relatively less virulent species, yielded 70.3 mg crude lipid g^{-1} dry weight of the cells whereas, 59.7 mg of crude lipid g^{1} dry weight of the cells was obtained from avirulent *L. seeligeri*.

Water- soluble fraction of the crude lipid extract of *Listeria* species contained protein, carbohydrate and unidentified some substances (Table-1).

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Table-1: Chemical analysis of the crude lipid extract of *Listeria* species grown At 37 °C in tryptone soya broth.

Bacteria	Total lipid extracted mg gm-1 dry weight of cells	Protein (%)	Carbohydrate (%)	Unidentified Substances (%)
L. monocytogenes (NCTC 7973)	**77.8**	**4.4**	**4.8**	**3.0**
***L. ivanovii* (SLCC 2379)**	**70.3**	**5.2**	**8.1**	**4.0**
L. seeligeri (SLCC 3954)	**59.7**	**N.D.**	**N.D.**	**N.D.**

N.D. = Not done, since *L. seeligeri* did not exhibit enhancement of monocyte count.

The crude lipid extract of *L monocytogenes* and *L. ivanovii* was found to be non-toxic, the LD_{50} being non-significant in the brine shrimp toxicity assay and no effect of lipid was observed on the newly hatched *Artemia* larvae at different doses. The LD_{50} was insignificant having chi square value 0.0285 at 1 degree of freedom, when calculated by Finney's method of bioassay.

The relative quantity of the major fatty acids (FAs) are given in Table-2. It is evident from date that as few as 3 or 4 major FAs dominate the lipid composition of *Listeria* species. The most common FAs of *Listeria* species grown at 37 °C in TSB were the branched (ante-iso) forms of the odd carbon chain.

The predominant odd chain FA in all the three species tested were branched anteiso-forms C 15:0 and C17:0 with smaller amount of iso-15 and iso-17 (Table-2). The next abundant FA was C 16 chain (anteiso).

Table-2: Fatty acids composition (relative percentage) of lipids extracted with $CHCL_3$-MEOH (2:1) from different *Listeria* species.

Fatty acids	*L. monocytogenes* NCTC 7973	*L. ivanovii* SLCC 2379	*L. seeligeri* SLCC 3954
12:0	**---**	**---**	**tr**
13:0	**---**	**---**	**tr**
iso 14	**---**	**1.70**	**9.80**
anteiso 14:0	**---**	**3.00**	**3.70**
iso 15	**7.10**	**15.00**	**12.50**
anteiso 15:0	**35.80**	**44.50**	**22.80**
15, ald*	**2.86**	**---**	**---**
anteiso 16:0	**10:00**	**14.61**	**12.69**
iso 17	**2.16**	**7.10**	**3.40**
anteiso 17:0	**5.29**	**10.70**	**29.70**
18-sat**	**8.60**	**4.00**	**---**
18-unsat***	**7.10**	**3.50**	**4.55**
iso 19:0	**3.50**	**---**	**---**
iso 22	**4.30**	**---**	**---**
Unidentified Substances	**2.00**	**3.00**	**2.00**

Values are the mean of duplicate fatty acid methyl esters.
* Aldehyde
** Saturated
*** Unsaturated
iso = methyl group at the ω -1 position.
Anteiso = methyl group at the ω -2 position.
Contents ≤ 0.1 % = tr.

Some species differences have been revealed from the results (Table- 2). Branched C 14:0 iso and anteiso- forms were present in *L.ivanovii* and *L. seeligeri* but was lacking in *L. monocytogenes* strains. Similarly straight chain FA C 12:0 and C 13:0 were present only in *L. seeligeri* though in trace amount. It was completely absent in the lipid of *L. ivanovii* and *L. monocytogenes*. Saturated and unsaturated iso-18:0 chain FAs were present in *L. ivanovii* and *L. monocytogenes* while only unsaturated iso-18:0 was present in *L. seeligeri*.

Interesting characteristic of *L.monocytogenes* NCTC 7973 FAs is the presence of iso-19 and iso-22 amount as evident in the Mass spectra of C 19 and C 22 FAMEs shown in Fig. 1 and Fig. 2.

Blood Picture

Fig. 3a and Fig. 3b show changes which occurred in the blood of animals. As shown in Fig. 3a killed *L. monocytogenes* cells provoked strong monocytosis after 48 PIH, while at the same time a slight variation was observed in the number of lymphocytes (result not indicated).

A marked increase in the circulating monocytes was observed in blood when live *L. ivanovii* cells were injected (Fig. 3b). The level reached at maximum after 72 hours. No significant change in the monocyte count was observed when live cells of *L. seeligeri* were injected (Data not shown)

Fig. 4 depicts the level of blood monocytes in response to the crude lipid extract of *L. monocytogenes*. Lipid dose was sufficient to cause a marked increase from 2% to 15% in blood monocytes level at 72 PIH in rabbits. A similar effect was observed in rates and mice except that in mice the monocyte level started increasing significantly after 48 PIH.

The crude lipid extract of *L. ivanovii* did not exhibit any reaction in the tested species of animals even after 72 PIH (data not shown), the time at which monocytosis usually reaches peak levels.

Cellular FA analysis technology is used as a tool for identifying and classifying microorganisms [17, 18]. The composition of the FA of bacterial cells is different in different species; even the species within the same genus *Listeria* represent a particular

identical FA pattern, which may not be equally virulent [19]. In the present study we obtained crude lipids as the chloroform-methanol extracts. Apart from lipids the extract also contained water-soluble fractions consisting of proteins, carbohydrates and some unidentified compounds. No significant difference exists in the protein and carbohydrate contents in the crude lipid extract of different *Listeria* species (Table-1). Any correlation between the chemical composition of the lipid and its biological activity is unexplainable at this stage.

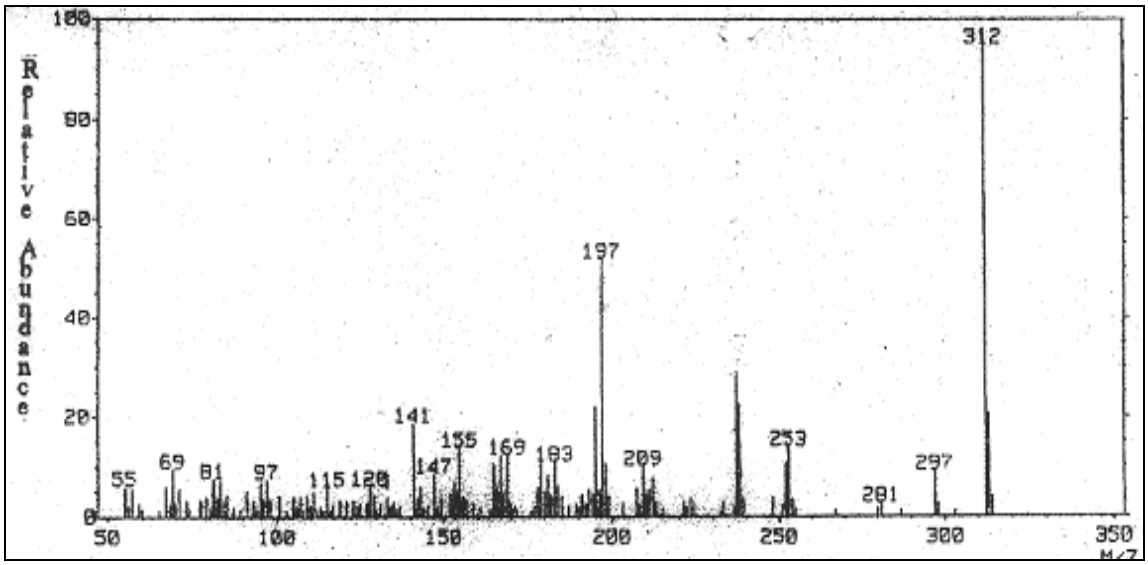

Fig. 1: Mass spectrum of C19 carbon chain fatty acid methyl ester from L. monocytogenes NCTC 7973 lipid, base peak m/e 74.

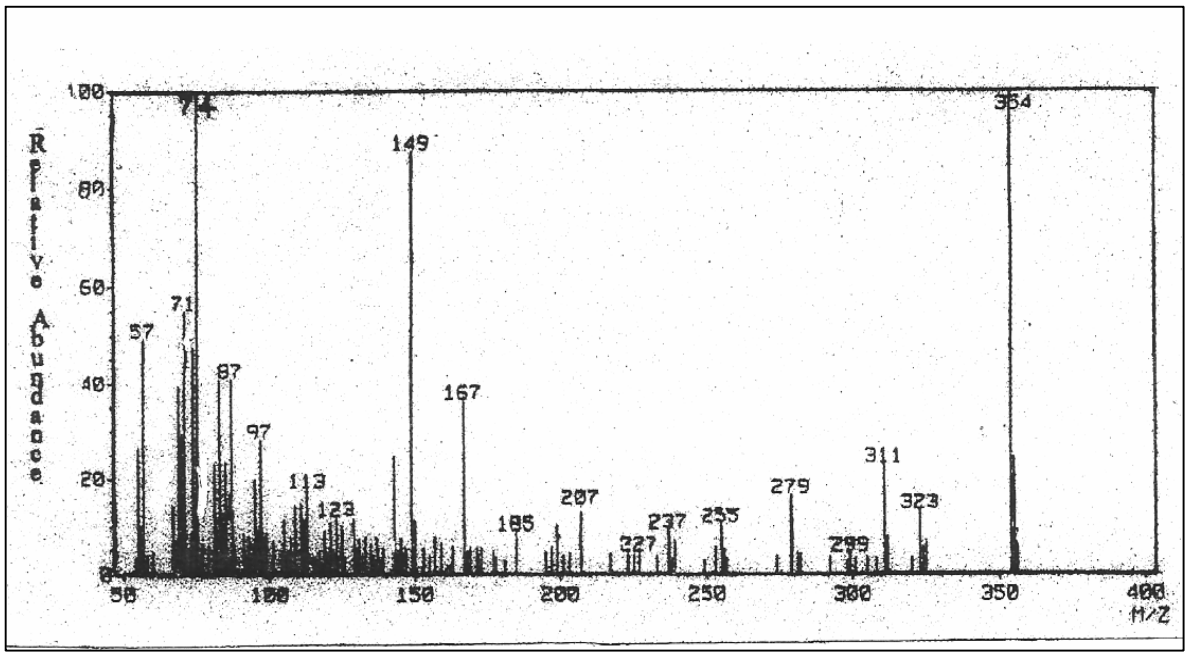

Fig. 2: Mass spectrum of C22 carbon chain fatty acid methyl ester from L. monocytogenes NCTC 7973 lipid, base peak m/e 74.

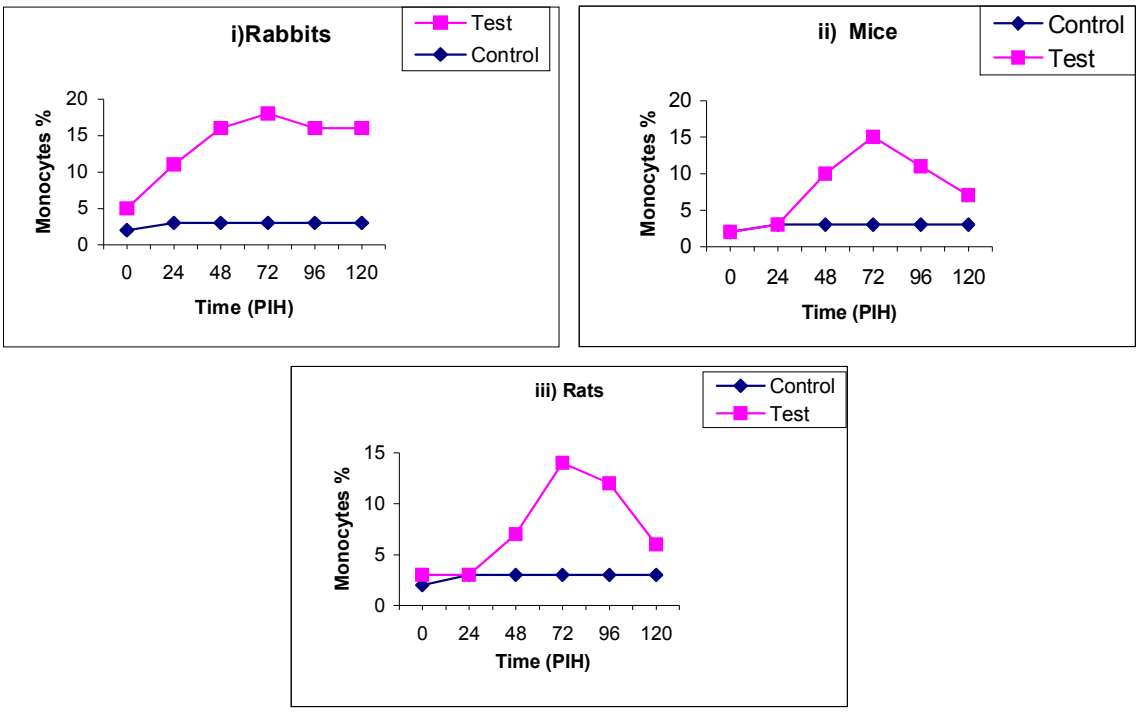

Fig. 3a: Effect of killed *L.monocytogenes* NCTC 7973 on peripheral blood monocytes number. Dose 1x10 ^{8}CFU mL $^{-1}$ (i) rabbits = 1mL (ii) mice=0.2ml, (iii) rats=0.5 mL Results expressed as arithmetic mean values from each set of animals. SEM ranges $\pm$ 0.02 to $\pm$ 0.38, No. of trials = 2.

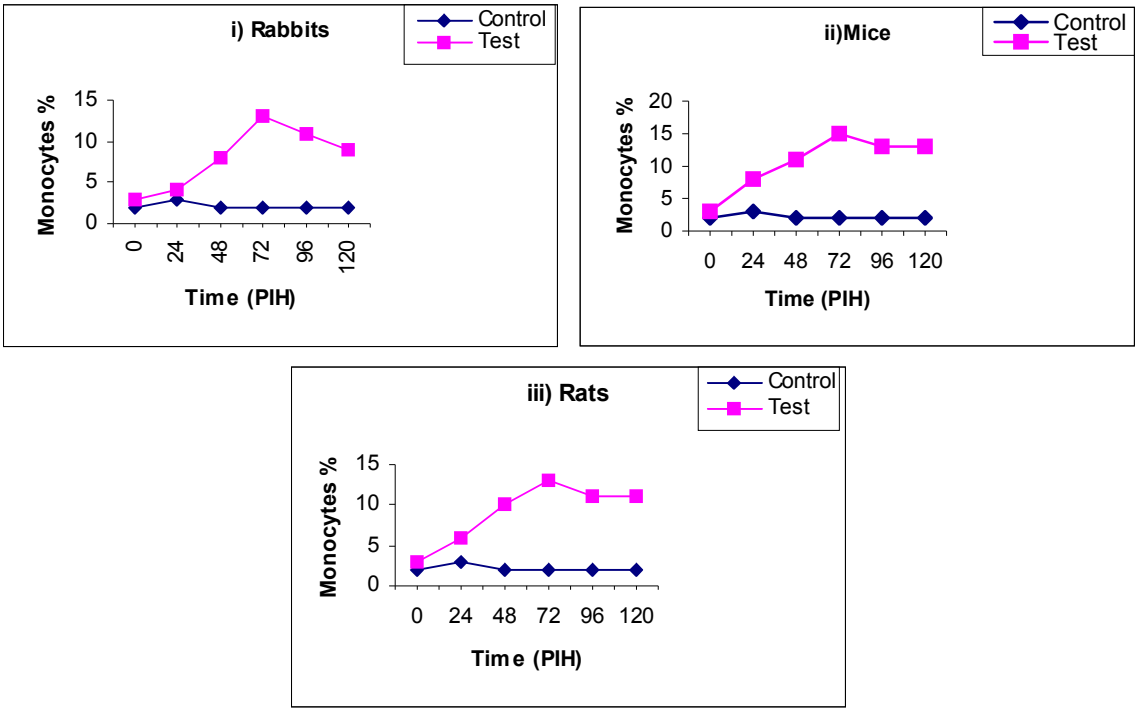

Fig. 3b: Effect of live *L.ivanovii* SLCC 2379 on peripheral blood monocytes number. Dose 1x 10 ^{8}CFU mL $^{-1}$ (i) rabbits = 1mL, (ii) mice=0.2mL, (iii) rats=0.5 mL results expressed as arithmetic mean values from each set of animals. SEM ranges $\pm$ 0.02 to $\pm$ 0.38, No. of trials = 2.

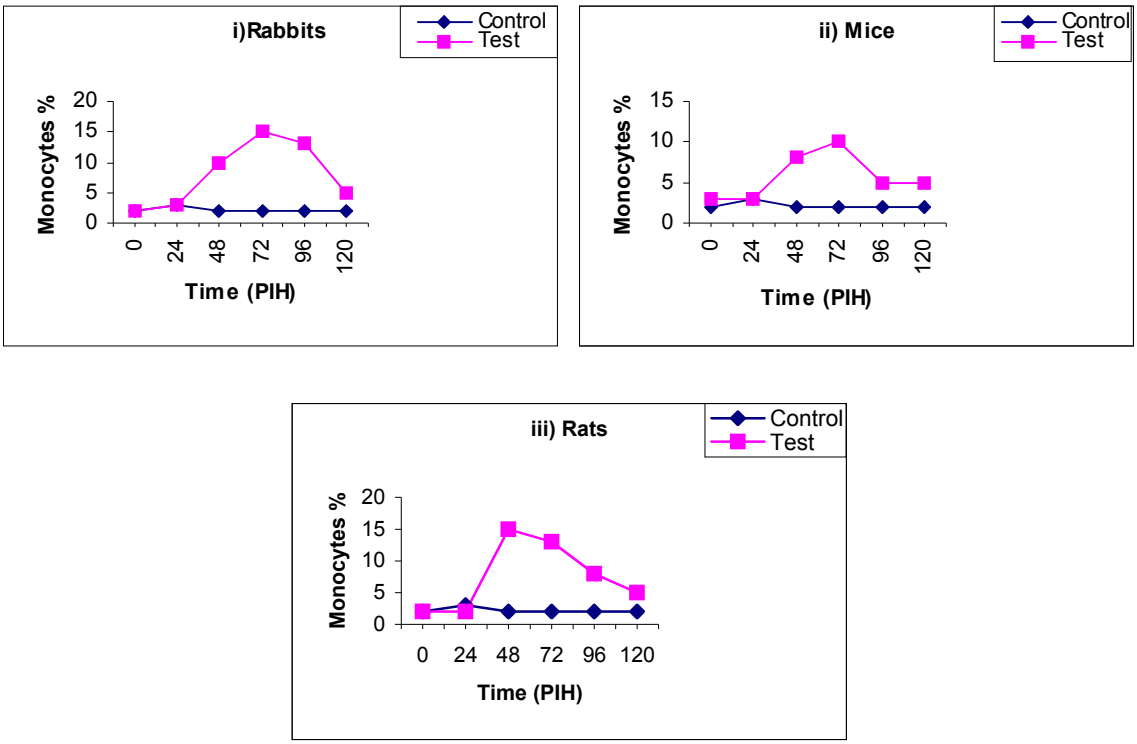

Fig. 4: Effect of crude lipid of *L mnocytogenes* NCTC 7973 on peripheral blood monocytes number. Dose (i) rabbits=20 mg mL^{-1} (ii) mice=2 mg $mL^{-1,}$ (iii) rats = 5 mg mL^{-1} results expressed as arithmetic mean values from each set of animals. SEM ranges $\pm$ 0.02 to $\pm$ 0.38, No. of trials = 2.

The chemical composition of the lipids of *Listeria* is characterized by the predominance of anteiso-C 15, C 16, C 17, and iso-C 18 FAMEs which is in agreement with previously published reports [19, 20, 21]. Of the studies that analysed the forms of branching the anteiso- forms, in particular anteiso 15 was more dominant than iso- forms. There are number of other FAs present in lower amount, depending upon the species and the environmental factors, importantly temperature [13, 15]. In the present study the C 14:0 FA was absent from the lipids of *L. monocytogenes,* in contrast to the previous reports [22]. The C19 and C22 FAMEs though present in low amounts seem to be characteristic of *L. monocytogenes*. The presence of C 19 and C 22 FA in *L. monocytogenes* reflects the presence of long carbon chain in the lipids of *Listeria* [23], which is its normal biosynthetic capability [24]. The variations in the relative amounts of these FA within *Listeria* may be used as a chemotaxonomic marker. However, no apparent correlation between this marker and the biological activity of the individual strains could be found.

It was observed that killed *L. monocytogenes* whole cells as well as their lipids has the ability to cause monocytosis in all three different species of animals. It has been reported that the killed *L. monocytogenes* and their lipids exhibit a high adjuvant activity [25]. It was also observed that the said characteristic is present in the whole cells of *L. ivanovii* whereas absent from its crude lipid extract, which seems to be significant. It could be argued that in order to obtain the above mentioned effect, the presence of particular FA moiety might be important. The results of avirulent *L. seeligeri* SLCC 3954 show that the monocytosis producing ability is totally absent in the whole cells both live and killed.It seems that some other extracellular products together with lipids contribute in the pathgenicity of *Listeria* which were however absent in *L.seeligeri*.

The above view is further supported by the fact that the greater quantity of MPA is associated with the comparatively virulent strains and little or no monocytosis producing agent with the less virulent or avirulent strains. The data further corroborates previous opinion that MPA is one of the factors responsible for the pathogenicity of the bacterium. The C-15 aldehyde and long chain C19 and C22 FA moieties along with some water soluble components (protein and carbohydrate), presents a specific

structural configuration, which may be responsible for MPA due to synergistic effect. However, we could not demonstrate any correlation between the chemical composition of the lipids of *Listeria* and biological activity.

The present study seems to be the first report on the MPA of the whole cells of *L. ivanovii*. Our finding that MPA is present only in the lipids of *L. monocytogenes* but absent from the lipids of *L. ivanovii* also sustains the view that serovar 5 of *L. monocytogenes* is a separate species

Experimental

Animals

The following experimental animals were used:
rabbits of both sexes ranging from 1500-1800 g in weight.
rats (Strain wister) weighing between 200-250 g.
mice (Strain NMRI) weighing 18-20 g.

Animals were kept under sanitary conditions, fed conventional diet and given water ad libitum.

Cultures

L. monocytogenes NCTC 7973, *L. ivanovii* SLCC 2379 and *L. seeligeri* SLCC 3954 were maintained on Tryptone Soya Agar (TSA, Oxoid) slopes at 4 °C and their identity was confirmed by microscopy, cultural characteristics and biochemical tests.

Biomass Production Biomass

Production of the above mentioned *Listeria* species in Tryptone Soya Broth (TSB) was carried out in a conical flask containing 3 litres inoculated broth which was incubated at 37 °C for 3 days. The cultures were then checked for purity, and heat killed by autoclaving. Cells were harvested by centrifugation at 6000 r.p.m. for 20 minutes and lyophilized after washing twice with phosphate buffer saline (PBS pH 7.0).

To demostrate the effect of whole cells on circulating monocytes, the cultures were grown on TSA slants for 24 h at 37 °C, washed twice with PBS (pH 7.0) and finally resuspended in PBS. The turbidity was matched with the McFarland index ($1x10^8$ cells ml^{-1}).The viability of cells was further confirmed by plating culture onto solid agar media. cells were killed by heating at 80 °C for 20 minutes *L.monocytogenes*.

Extraction of Lipids

The extraction of lipids from the lypohilized dead cells was performed by using a mixture of chloroform-methanol (2:1) v/v in a Soxhlet apparatus for two days.

Preparation of Fatty Acid Methyl Esters (FAMEs)

The extract was dried under vacuum, washed with water, re-extracted using chloroform and then filtered. The filtrate was again dried under vacuum. The residue thus obtained was saponified by using methanol in 10% KOH and refluxed for 24 h. The resulting mixture of the salts of carboxylic acids was first acidified with 1M HCl, extracted with ethyl acetate and then esterified with methanol/sulphuric acid by heating under reflux for 4 h. Finally the reaction mixture was made alkaline (pH 11.0) using 1M NaOH and the corresponding methyl esters were extracted with diethyl ether after removing excess of methanol on a rotary evaporator. The lipid material was dissolved in minimal volume of diethyl ether and transferred to preweighed vials. For determining total extracted lipid, samples were first dried to constant weight on a rotary evaporator and then in a vaccum oven at 50 °C [26].

The FAMEs were analyzed by gas-chromatography-mass spectroscopy (GC-MS) on a HP-5890, JMSH x 100 (JEOL, japan) 30 M X 0.25 mm (i.d.) column, carrier gas heater. The column temperature was programmed between 200-260 °C with the rate of increase of 5 °C/min., ionization voltage was 70 eV, total emission current was 100 μ A. The peaks were identified by using mass spectral data index, further confirmed by a comparison of retention times with known standards (Sigma).

Analytical Assay

The water soluble fraction of the crude extract was extracted using separating funnel. The protein and carbohydrate contents in the crude water extract were determined by the method of Lowry *et al.* [27] and by the anthrone method of Kahan [28], respectively.

Toxicity Evaluation

The toxicity of the crude lipid extract was determined by the brine shrimp assay [29].

Assay of MPA

MPA of the whole cells of *Listeria* (killed and live) species was determined by injecting 1, 0.5 and 0.2 mL of 1x 10^8 cells mL^{-1} in rabbits, rats and mice respectively. All injections in rats were made intraperitonealy, in rabbits *via* peripheral ear vein and in mice through tail vein.

The same inoculation pattern was follwed for determining the MPA of crude lipid extract. The sample was prepared by emulsifying the vacuum dried lipid extract in a diluent (0.9% NaCl containing 0.3% sodium lauryl sulphate). Lipid doses were 20 mg mL^{-1}, 5 mg mL^{-1} and 2 mg mL^{-1} in rabbits, rats and mice, respectively. In each set of experiments the control animals received only diluent.

Monocyte Count

Blood samples were obtained immediately before injection (0 hour) and then at 24, 48, 72, 96 and 120 post injection hours (PIH). Samples of peripheral blood were obtained by incision of tail vein in the case of mice and rats and from marginal ear vein in rabbits. A thin blood film on a glass slide was prepared on a glass slide, stained with Giemsa's stain and the Differential Leukocyte Count (DLC) was determined.

Statistical Analysis

Experimental results were grouped according to treatment and the arithmetic average was calculated for each group for the values for each individual within that group. This average was expressed as the mean (X) plus or minus (±) the standard error of the mean (SEM), with the number of subjects (N). The SEM was calculated by the N-1 method for obtaining an unbiased estimate of the population variance. The significance was calculated by the t-test wherever necessary.

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