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Growth Inhibitory Effect of 2-Nitropropanol on Foodborne Bacteria of Public Health Importance

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A B S T R A C T

The investigation was aimed at to proximate the minimum inhibitory concentration (MIC) of 2-nitropropanol (2-NPOH) for growth inhibition (*in-vitro*) of *Salmonella typhimurium*, Shigatoxic *Escherichia coli* (STEC) and *Salmonella gallinarum vis a vis* effect of pH of culture medium on function of 2-NPOH and its *in-vivo* effect on these bacteria in chick model. In *in-vitro* testing, MIC of 2-NPOH for *Salmonella typhimurium* was recorded to be 3.5 mM whereas it was 1.5 mM for STEC and *Salmonella gallinarum*. These organisms were sensitive to acidic pH of the growth medium. Increasing pH of the medium *viz.* 7.3, 8.0, 8.5, 9.0 and 9.5 (without adding 2-NPOH) revealed that higher alkalinity did not affect growth status of these organisms. On the analogy, growth medium supplemented with sub-MIC dosage of 2-NPOH in different alkaline pH (8.0, 8.5, 9.0 and 9.5) although exerted its bacteriostatic effect; but had no significant variation on growth at different pH (> 8.0). *In-vivo* effect of 2-NPOH in chick model (4 days old) revealed that its MIC dosages may considerably reduce carriage of intestinal bacteria like *Salmonella typhimurium* ($26x10^7$ to $10.5x10^6$ cfu/gm of faeces), STEC ($33x10^7$ to $11.2X10^6$cfu/gm faeces) and *Salmonella gallinarum* ($29x10^7$ to $9.4x10^6$cfu/gm of faeces). Above all, the findings suggested that 2-NPOH may be used as substitute of antibiotic to reduce the carriage of gut microbiota of public health importance in poultry.

Introduction

Despite of adopting recommended measures and intensive precautions for prevention of contamination in food processing, storage and supply; foodborne outbreaks in human continue to occur (Yang *et al*., 2017). CDC estimates that each year 48 million people get sick from a foodborne illness, 128,000 are hospitalized, and 3,000 die in the U.S. (CDC, 2018). Annual summary of CDC Report documented in 2016 reveals a total of 839 foodborne disease outbreaks, resulting in 14,259 illnesses, 875 hospitalizations, 17 deaths, and 18 food recalls wherein Salmonella, STEC, *Clostridium perfringens* and Norovirus were detected to be the leading pathogens. Further, in 2010 there was report of 600 million of foodborne illness globally and 4,20,000 deaths where the most frequent causes of foodborne illness were diarrhoeal disease agents, particularly Norovirus and

Campylobacters followed by non-typhoidal *Salmonella enterica*, and also *Salmonella typhi*, *Taenia solium*, hepatitis A virus, and aflatoxin (WHO, 2015). An estimated annual 300,000 hospitalizations and 5000 deaths in the U.S. alone are related to food-borne illnesses; which reflects economic implications costing the US economy more than $15.6 billion (USDA, 2014). The economic loss of food poisoning outbreaks in developing country like Indonesia in 2013 was approximately US$ 78 million (Rahayu *et al*., 2016).

Efforts to reduce the carriage of the intestinal pathogens in food animals have been made in veterinary medicine by use of antibiotics; however, this induces the emergence of antibiotic resistant bacterial strains that pose a burgeoning burden in public health (Hao *et al*., 2014). To avoid this, in recent past, the approach of using innovative strategies such as prebiotic, probiotic, immunomodulators, bacterial growth inhibition by various chemicals/ constituents, bacterial antagonism by saprophytes etc. have gained momentum as substitute to antibiotics (Strompfova and Laukova, 2007). The bacteriocin 'Enterocin AS-48' produced by Enterococcus is widely used in packed food industry as a natural food bio-preservative against a list of food borne and food spoilage pathogens (Grande Burgos *et al*., 2014; Ortega *et al*., 2018). Reduction of harmful aerobic intestinal bacteria (having the nitrate reductase enzyme) in food animals using the chlorate compound is also a promising approach (Anderson *et al*., 2005) where aerobic bacteria by virtue of their respiratory nitrate reductase enzyme can metabolize nitrate that in turn converts inorganic chlorate to cytotoxic chlorite. Interestingly, most of the beneficial anaerobic gut bacteria lack respiratory nitrate reductase activity and escape the experimental chlorate cytotoxic action. Thus chlorate may selectively target those gut bacteria possessing a respiratory nitrate reductase enzyme (mostly aerobes) but not the beneficial anaerobes lacking the enzyme; thereby, conserving the competitive exclusion potential of the host's normal flora (Anderson *et al*., 2000). Many efforts have been made for reduction and possible elimination of pathogens during all stages of production in order to prevent the foodborne problems (Jung *et al*., 2004; Anderson *et al*., 2005); however, none of the measures alone stands as absolutely effective.

Ability of potential probiotic strains to adhere to the intestinal mucosa and exclude & displace pathogens is of utmost importance for therapeutic manipulation of the enteric microbiota (Cassir *et al*., 2016). Besides, literature review suggests that different group of researchers have studied with different chemicals like nitrate and chlorate (Jung *et al*., 2003; Anderson *et al*., 2005), 2-nitro-1-propanol & 3 nitro-1-propanol (Jung *et al*., 2004), 2-NPOH along with 2-nitroethanol (Adhikari *et al*., 2016; Adhikari *et al*., 2017) to explore their effects to reduce the carriage of intestinal pathogens as well as to evade the use of antibiotics and thereby, emergence of resistant strains. Although limited researches have been carried out in this line in abroad; however, no such synchronised study, so far, has been recorded in Indian context. Considering the back ground information, the present investigation was aimed at to evaluate the potentials of 2-NPOH as an alternative of antimicrobial supplement in food chain in order to minimize the foodborne problems with commonly occurred aerobic bacteria viz. *Salmonella typhimurium* and STEC.

Materials and Methods

Reference culture

Reference cultures of *Salmonella typhimurium* and STEC were obtained by kind courtesy of Department of Microbiology, National

Institute of Cholera and Enteric Diseases (NICED), Kolkata, India. Considering the host animal's susceptibility and suitability, *Salmonella gallinarum*, included as reference culture to assess the *in-vivo* effect of 2-NPOH in chick, was obtained from ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, U.P., India.

Determination of minimum inhibitory concentration (MIC) of 2-NPOH (*in-vitro*)

Molar solution of 2-NPOH was prepared in triple distilled water and made sterile by filtering through syringe filter (Milipore; 0.22 µm) on the laminar flow. Calculated volume of such 2-NPOH solution was added to pre-autoclaved Tryptic Soy Broth (TSB) to make different molar final concentration (1.0; 1.25; 1.5; 2.0; 2.5; 3.0, 3.25 and 3.5 mM) of 2-NPOH in TSB and the prepared TSB were kept overnight at 37ºC to check sterility. On the next day, the bottle of TSB (found no growth) was inoculated with the target bacterial culture and observed for growth (turbidity and test plate culture) at 6, 12, 24, 48, 96 and 120 hrs of incubation. The TSB supplemented with 2-NPOH and exhibited no growth up to 120 hrs was kept under observation up to two weeks to check growth, if any, of inoculated bacteria. MIC of 2-NPOH for *in-vitro* growth inhibition of *Salmonella gallinarum* was assessed in this study for evaluation of its *in-vivo* effect in chicks.

Isolates of STEC (n=12) and *Salmonella gallinarum* (n=5) obtained from field samples were assessed (*in-vitro*) for effect of 2-NPOH following the same method as mentioned above for reference culture.

Effect of 2-NPOH on growth logarithm of test cultures

To understand the effect of 2-NPOH on growth logarithm of the test bacteria, the cultures were grown in medium without 2-NPOH and vis-à-vis with use of 2-NPOH (sub- MIC dosages) and colony count was recorded in different span of incubation (6 hrs, 12 hrs and 24 hrs).

Effect of pH of culture medium on growth logarithm of test culture

To know the effect of pH on growth of the test organisms, pH of the medium (TSB) without supplement of 2-NPOH was adjusted to 5.0, 6.0, 6.5, 7.3 (dehydrated medium pH), 8.0, 8.5, 9.0 and 9.5 and were incubated on inoculation with the test organisms.

Findings for growth of the test culture in TSB (without 2-NPOH) indicate that none of the test culture grow in the medium having pH below 7.3. Therefore, TSB was prepared with pH 7.3, 8.0, 8.5, 9.0 and 9.5 to assess the effect of 2-NPOH in different alkaline pH. Further, such growth medium was supplemented with sub-MIC doses of 2-NPOH to evaluate the bacteriostatic effect of 2-NPOH in variable pH of growth medium.

In vivo effect of 2-NPOH in chick model

In-vivo effect of 2-NPOH was studied using 4 days old chick in different groups [control (1) and study groups (4)]. Each group consisted of 6 birds. At the beginning, total bacterial count (TBC) per gram of faecal content was assessed in the birds of control as well as first study group (where no 2-NPOH was used) by serial dilution of faeces in peptone water followed by spread plating onto the Mueller Hinton agar (MHA) (average of count was considered as value). Likewise, 2-NPOH (MIC dose) was fed in the first study group and TBC per gram of faeces was assessed (average of count was considered as value) after 24 hrs. Intestinal bacterial load was counted in the second study group and thereafter, fed with young culture of STEC

grown overnight in TSB (~10^6 cfu), and TBC per gram of faeces was recorded after 24 hrs. Then, 2-NPOH (MIC dose) was given orally to this study group and TBC per gram of faeces was noted after 24 hrs.

Similar to the second study group, chicks in the third study group were fed with ~10^6cfu of novobiocin (25 µg) and nalidixic acid (20 µg) resistant *Salmonella typhimurium* culture grown overnight in TSB and TBC per gram of faeces was recorded after 24 hrs. Thereafter, 2-NPOH (MIC dose) was given orally to this study group and TBC per gram of faeces was noted after 24 hrs by diluting the collected faeces from such birds in peptone water and thereafter, spreading onto the Brilliant Green Agar (BGA) supplemented with novobiocin (25 µg/ml) and nalidixic acid (20 µg/ml) to enumerate *Salmonella typhimurium*. Similarly, such assessment of 2-NPOH was also done for *Salmonella gallinarum* in fourth study group.

Results and Discussion

On *in-vitro* assay for determination of MIC of 2-NPOH, it was observed that 3.5 mM of 2-NPOH was the MIC for *Salmonella typhimurium* and 1.5 mM for STEC and *Salmonella gallinarum* (Table 1).

In the growth medium free from 2-NPOH, *Salmonella typhimurium* exhibited 7x10^6 cfu/ ml, 20x10^6 cfu/ml and 41x10^6 cfu/ml in 6 hrs, 12 hrs and 24 hrs of incubation, respectively (Table 2). Likewise, for STEC, 20x10^6 cfu/ml, 65 x10^6 cfu/ml and 170x10^6 cfu/ml was recorded in 6 hrs, 12 hrs and 24 hrs of incubation. Similarly, for *Salmonella gallinarum*, it was 11x10^6 cfu/ml, 35x10^6 cfu/ml and 150x10^6 cfu/ml, respectively in 6 hrs, 12 hrs and 24 hrs of incubation (Table 2).

When *Salmonella typhimurium* was allowed to grow in the medium supplemented with immediate sub MIC (3.0 mM) of 2-NPOH (MIC is 3.5 mM), it revealed 70x10^3 cfu/ml, 1.7x10^6 cfu/ml and 2.5x10^6 cfu/ml, respectively in 6 hrs, 12 hrs and 24 hrs of incubation (Table 2). Similarly, the STEC in its immediate sub-MIC (1.0 MM) of 2-NPOH revealed 1.4x10^6 cfu/ml, 2.4 x10^6 cfu/ml and 3.5 x10^6 cfu/ml in 6 hrs, 12 hrs and 24 hrs of incubation (Table 2). Likewise, for *Salmonella gallinarum*, it was 75x10^3cfu/ml, 1.5 x10^6 cfu/ml and 2.7x10^6 cfu/ml, respectively in 6 hrs, 12 hrs and 24 hrs of incubation (Table 2). The observation focused that the growth rate of the above three bacterial species was significantly reduced with the use of 2-NPOH in its sub-MIC. It appears that even in sub-MIC doses, 2-NPOH facilitates its bacteriostatic property on the tested foodborne bacterial pathogens.

The present findings support the observations of earlier study by Jung *et al.,* (2004). Since, few published works are available on this aspect, the present findings could not be thoroughly discussed with comparison. The study observation is in accord to the trend of result in the experiment by Jung *et al*., (2004) and Adhikari *et al*., (2016) where they have recorded considerable growth inhibition effect at variable concentration (mM) of 2-NPOH on *Salmonella typhimurium* and *E. coli* O157:H7 (Shigatoxic).

In order to know the effect of variable pH of growth medium on the test organisms, pH of the culture medium was adjusted to 5.0, 6.0, 6.5, 7.3 (pH of the dehydrated medium), 8.0, 8.5, 9.0 and 9.5 and the test organisms were allowed for incubation (Table 3). It was noted that none of the 3 bacterial cultures grew in the culture medium (TSB) where pH was maintained below 7.3. The result suggested that these organisms are sensitive to acidic pH and the effect of 2-NPOH on these organisms could be assessed adopting the standard media pH 7.3 and above.

Table.1 Minimum inhibitory concentration (MIC) (in vitro) of 2-NPOH

	Hours of incubation					
	Salmonella typhimurium					
Molar Concentration of 2-NPOH	6	12	24	48	96	120
1 mM	+	+	+	+	+	+
1.5 mM	+	+	+	+	+	+
2 mM	+	+	+	+	+	+
2.5 mM	+	+	+	+	+	+
3.0 mM	+	+	+	+	+	+
3.5 mM	-	-	-	-	-	-
3.25 mM	-	-	+	+	+	+
	STEC					
1 mM	-	+	+	+	+	+
1.5 mM	-	-	-	-	-	-
1.25 mM	-	-	+	+	+	+
	Salmonella gallinarum					
1 mM	-	+	+	+	+	+
1.5 mM	-	-	-	-	-	-
1.25 mM	-	-	+	+	+	+

(-)= no growth; (+)= presence of growth.

Table.2 Effect of 2-NPOH on aerobic growth rate of test bacterial culture

	Hours of incubation		
2-NPOH Concentration	6	12	24
Salmonella typhimurium			
2-NPOH not added.	$7x10^6$ cfu/ ml	$20x10^6$ cfu/ml	$41x10^6$ cfu/ml
3.0 mM	$70x10^3$ cfu/ml	$1.7x10^6$ cfu/ml	$2.5x10^6$ cfu/ml
STEC			
2-NPOH not added.	$20x10^6$ cfu/ml	$65 x10^6$ cfu/ml	$170x10^6$ cfu/ml
1.0 mM	$1.4x10^6$ cfu/ml	$2.4 x10^6$ cfu/ml	$3.5 x10^6$ cfu/ml
Salmonella gallinarum			
2-NPOH not added.	$11x10^6$ cfu/ml	$35x10^6$ cfu/ml	$150x10^6$ cfu/ml
1.0 mM	$75x10^3$ cfu/ml	$1.5 x10^6$ cfu/ml	$2.7x10^6$ cfu/ml

Table.3 Effect of variable pH of growth medium on the test organisms

Organism	**pH**							
	5.0	6.0	6.5	7.3	8.0	8.5	9.0	9.5
Salmonella typhimurium	-	-	-	+	+	+	+	+
Shigatoxic *E. coli* (STEC)	-	-	-	+	+	+	+	+
Salmonella gallinarum	-	-	-	+	+	+	+	+

Table.4 Effect of 2-NPOH on growth* of the bacteria in medium with different pH

	pH				
2-NPOH Concentration	7.3	8.0	8.5	9.0	9.5
	Salmonella typhimurium				
2-NPOH not added	$20x10^6$	$19x10^6$	$19x10^6$	$19x10^6$	$20x10^6$
3.0 mM	$1.75x10^6$	$1.70x10^6$	$1.75x10^6$	$1.70x10^6$	$1.70x10^6$
STEC					
2-NPOH not added	$65x10^6$	$64x10^6$	$65x10^6$	$64x10^6$	$65x10^6$
1.0 mM	$2.6\ x10^6$	$2.6x10^6$	$2.4x10^6$	$2.6x10^6$	$2.6x10^6$
	Salmonella gallinarum				
2-NPOH not added	$35x10^6$	$35x10^6$	$35x10^6$	$34x10^6$	$35x10^6$
1.0 mM	$1.55x10^6$	$1.65\ x10^6$	$1.50\ x10^6$	$1.55\ x10^6$	$1.50\ x10^6$

*Unit of bacterial growth is cfu/ml; Incubation hour- overnight.

Therefore, bacteriological culture was set in TSB having different alkaline pH (7.3, 8.0, 8.5, 9.0 and 9.5) and supplemented with 2-NPOH with a concentration of immediate below MIC molarity *viz*. for *Salmonella typhimurium* (3.0 mM 2-NPOH); STEC & *Salmonella gallinarum* (1.0 mM 2-NPOH).

It was observed that overnight broth culture of *Salmonella typhimurium* grown in the medium (without 2-NPOH) with pH 7.3, 8.0, 8.5, 9.0 and 9.5 yielded 20 $x10^6$ cfu/ml; however, when such medium was supplemented with 3.0 mM 2-NPOH (MIC is 3.5 mM) the recovered growth was from $1.7X10^6$ cfu/ml to 1.75 $X10^6$ cfu/ml (Table 4). Similarly, STEC exhibited 65 $x10^6$ cfu/ml in 2-NPOH free medium of different alkaline pH; however, growth population was reduced to a range of 2.4 $x10^6$ cfu/ml to 2.6 $x10^6$ cfu/ml when allowed to grow in the medium supplemented with 1.0 mM 2-NPOH (MIC is 1.5 mM) (Table 4). Likewise, growth population of *Salmonella gallinarum* propagated (35 x 10^6) in 2-NPOH free medium of different alkaline pH and growth population reduced to a range of 1.5 $x10^6$ cfu/ml to 1.65 $x10^6$ cfu/ml (Table 4) in the medium supplemented with 1.0 mM 2-NPOH (MIC is 1.5 mM). The findings revealed the bacteriostatic effect of 2-NPOH in its sub-MIC dose, even in variable alkaline pH of growth medium.

To know the variation, if any in growth inhibitory effect (*in-vitro*) of 2-NPOH on the field isolates of the test bacteria; the assay of MIC was performed in field isolates of STEC (n=12) and *Salmonella gallinarum* (n=5) where observations were recorded in similar line to reference culture.

In the *in-vivo* assay, TBC in fresh chick was recorded to be 23 x 10^6 per gm of faeces and reduced to 14.6 x 10^6 cfu on use of 2-NPOH (MIC dosage). While young grown culture of

STEC was fed to a fresh study group of chicks, the TBC was recorded as $33x10^7$ cfu per gram of faeces. Subsequently, on oral feeding of 2-NPOH (MIC dosage) in this study group, TBC was noted to be reduced to $11.2\ x10^6$ cfu per gram of faeces.

Similarly, for challenge study with *Salmonella typhimurium*, the TBC was reduced from $26x10^7$ to $10.5x10^6$ cfu per gram of faeces. Likewise, for *Salmonella gallinarum*, the TBC was reduced from $29x10^7$ to $9.4\ x\ 10^6$ cfu per gram of faeces. The present study findings indicated that 2-NPOH in its MIC dosage in chick facilitates its growth inhibitory effect on STEC, *Salmonella typhimurium* and *Salmonella gallinarum* and reduced the carriage of the intestinal bacteria at a significant level. Above all, the study observations suggested that 2-NPOH may be used as an alternative of scheduled antibiotics supplemented in poultry feed in order to reduce the intestinal carriage of common aerobic bacteria of public health importance.

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