Eeffect of *Bifidobacterium longum* and Allyl isothiocynate on survival of *Salmonella typhimurium* in Fermented Sausage

Asmaa, M. Sh. Fayed; Ghada, S.E. Salem and Salwa, R. S. Hegazy

Food Hygiene Department, Animal Health Research Institute, Agricultural Research Center, Giza – Egypt
Ghada_m999@yahoo.com

Abstract: Two batch of fermented sausage were made: one for organoleptic examination and second one for Bacteriological examination, each batch were divided into four groups. Sausage batters were inoculated with two commercial starter culture organisms (7 log CFU/ g *Lactobacillus acidophilus* and *Sacromyces cerevisiae*). Allyl isothiocynate (AITC) was added to the two batters at 500 ppm. *B.longum* (10^7 log/g) was added in together with AITC or separately. *S. typhimurium* was added to the second batch to yield 6.3 log cfu/g. Sausages were fermented at 22°C for 5 days, subsequently sausages were dried at 18 °C for 7 days then stored at -4 °C. *S. typhimurium* count was monitored during fermentation, drying and storage. Count were reduced to undetectable level after 12 days of processing in sausages contain AITC and *B. longum*, while not detected in sausage contain AITC alone after 19 days of processing. Sausages contain *B. longum* alone and samples without additives *Salmonella* count reduced to undetectable level after 47 days from processing. Sensory evaluation of sausage showed that samples contain 500 ppm of AITC was considered the most acceptable one, although slightly spicy by panelists.
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1. Introduction:

Fermentation and drying are among some of the first methods used to preserve foods for extended storage. Fermented dry or semi-dry sausages (FDSS) are produced by fermenting and drying a raw meat batter containing sugar, seasonings/ spices, and/or curing agents. The fermentation is conducted by natural micro flora in the ingredients and/or by added starter cultures. The need for better control of food borne pathogens has been paramount in recent years. Probiotics are defined as "living micro-organisms which, upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition" **(Guarner and Schaafsma, 1998).**

Most probiotic microorganisms belong to the lactic acid bacteria (LAB) group, such as Lactobacillus spp., and Enterococcus spp., or to the genus *Bifidobacterium* **(Klein *et al.,* 1998).** Considerable interest has been developed to the use of *Bifidobacteria,* as natural bio-preservatives in food. *Bifidobacteria* have the ability to suppress the growth of pathogenic bacteria by producing organic acids **(Ibrahim and Bezkorovainy, 1993)** and other antimicrobial compounds such as bacteriocins **(Gibson and Wang, 1994, Gomes and Xavier Malcata, 1999; Ibrahim and Salameh, 2001).** Bifidobacteria also play an important role in human health as they have different health and therapeutic benefits such as the reduction of lactose intolerance, blood cholesterol level and colon cancer generation **(Wu *et al.,* 2011).** Because of these health effects of *Bifidobacteria*, continued interest in these bacteria has brought about their utilization in a Varity of foods. There are different studies to optimize the industrial production of food products containing *Bifidobacteria* to introduce a viable population of them into gastrointestinal tract **(Misharina *et al.,* 2008).**

More than 1000 deaths occur each year due to *Salmonella* infection, making *Salmonella* the deadliest food borne pathogen **(Mead *et al.,*1999)**. *Salmonella* spp may contaminate meat products via contaminated raw meat, ingredients and/or processing equipment, and/or from post-processing contamination. These pathogens have also been detected in raw meat and have also been shown to survive certain sausage manufacturing processes **(Glass and Doyle, 1989).**

Allyl isothiocynate, because of its natural origin (a natural compound in plants belonging to the family Cruciferae as horseradish and mustard), and being generally recognized as safe **(Code of Federal Regulations, 1999)** attracting more attention as a potential antimicrobial agent in food **(Lin *et al.,* 2000a&b).** AITC is present as a precursor in the form of the glucosinolate "sinigrin" in these natural sources. Sinigrin is hydrolyzed by the endogenous enzyme myrosinase to yield allyl and other isothiocynates **(Clydesdale, 1999)**. Allyl isothiocynate was reported to act by affecting protein structure of the bacterial cell through the cleavage of disulfide bonds (**Kawakishi and Kaneko, 1987).**

The objective of this study was to investigate the individual and compined antimicrobial effects of Allyl isothiocynate and *bifidobacterium longum* against *Salmonella typhimurium* inoculated in fermented dry sausages.

2. Materials and Methods:-

2.1. Bacterial strains:-

Strain of *Salmonella typhimurium* ATCC 14028 was obtained from Food Hygiene Department, Animal Health Research Institute. The culture was incubated at 37◦C for 24 hrs. Brilliant green agar (Difco) used for enumeration the organism by incubation at 37°C for 24 hrs. A commercial *Lactobacillus acidophilus* and *Sacromyces cerevisiae* (formula 10^8 and 10^7 respectively; Biobos biodistech) and *Bifidobacterium longum* (formula 10^8; Biobos biodistech) was prepared as per the manufacturer's instructions.

2.2. Allyl Isothiocynate:-

Allyl Isothiocynate, 95% (ALDRICH chemistry; cat:37,743-0).

2.3. Preparation and inoculation of sausage:-

Raw ground beef (20% fat) was obtained from local retail store and kept frozen until used. Sausage was prepared by mixing 5 Kg of raw ground beef, 1.9% sodium chloride, 120 ppm sodium nitrate, (Sigma chemical co., st. Louis, Mo), 95% cumin, 0.42% paprika, 0.42% black pepper and 0.25% dextrose (Difco laboratories inc., Detroit, MI). Following the mixing, the batter was inoculated with *Lactobacillus acidophilus* and *Sacromyces cerevisiae* to achieve a cell concentration into the batter of 10^8 and 10^7 respectively in the whole batter, then batter was divided into two portions, *Bifidobacterium longum* was added to only one portion as a co starter in concentration of 10^7 into the batter. *Salmonella typhimurium* was inoculated to the half of each portion to reach a cell concentration of 6 log $_{10}$CFU\gm sausage batter. Allyl Isothiocynate was added in a concentration of 500 ppm to the half of each meat portion. The batter potions were stuffed into natural large diameter beef casing, hand tied with cotton strings at 15 cm intervals. Each sausage link was clearly labeled (whether contain *Lactobacillus acidophilus* and *Sacromyces cerevisiae* alone or in together with *Bifidobacterium longum*, inoculated with *Salmonella typhimurium* or not and contain Allyl Isothiocynate or not). Sausages were hung vertically in an environmentally controlled incubator for fermentation at 22°C for 5 days, the sausages were then dried at 18◦C for 7 days then stored at -4°C. Sausages were sampled for microbial counts during fermentation and drying and at days 0, 7, 14, 21, 30, 49, 63, 77 during storage.

2.4. Microbial analyses:-

At each sampling intervals, a 25 g portion of the sausages from the middle of each stick was removed for the enumeration of cell counts of *Salmonella typhimurium*. The samples were transferred to a stomacher bag added with 45 ml of 0.1% sterile peptone water and mixed in a bag mixer stomacher (Stomacher Lab. Blender 400, Seward Lab. Serial no. 30469 type BA 7021, London). For 2 mins. Pathogen populations were enumerated by serial dilution in 0.1% sterile peptone water as needed and spread-plating 0.1 ml onto XLD agar (Difco). Plates were incubated at 37◦C for 24 hrs.

2.5. Data analyses:-

The values of reduction in viable counts, $\log_{10}$ reduction of *Salmonella typhimurium* from each trial were plotted versus time of fermentation, drying and storage. Log_{10} reductions were analyzed using the regression procedure of the Statically Analyses System (SAS) version 9.1 software for windows (SAS Institute inc., Cary, NC).

2.6. Organoleptic evaluation:-

According to ***Anna (1998)*** by using the 9-point hedonic scale. Flavor ***(Grossklaus et al., 1979 and Miller, 1994).*** Odor ***(Gracey, 1986 and Miller, 1994).*** The color and flavor of the collected samples were examined according to **Anna (1998)** by using the 9-point hedonic scale.

3.Results and Discussion

Sensory evaluation:

Odor and flavor are important aspects of meat quality, sometimes used as determining criteria in the acceptance or rejection of the product **(Mottram, 1994).** It is evident from the results in Figs. (1, 2) that the odour and flavor of the examined fermented sausage samples were (8.2, 9) in sausage sample(S_3) contain *L. acidophilus* (7 log CFU/ g) + allyl isocyothinat oil 500 ppm, while in sausage sample (S_4) contain *L.acidohpilus* (7 log CFU/ g) + *B.longum* 10^7 CFU/g + allyl isocyothinat oil 500 ppm were (7.5, 7.8), in sausage samples (S_1) contain *L.acidohpilus* (7 log CFU/ g) were (7, 7.3), and sausage samples (S_2) contain *L.acidohpilus* (7 log CFU/g) + *B.longum* 10^7 CFU /g were (6.3, 6.0), respectively.

The results achieved in Fig. (3) Indicated that the mean value of overall score were 8.6, 7.6, 7.1and 6.1 respectively in fermented sausage samples (S_3), (S_4), (S_1), (S_2). These results indicated that the mean overall scores of fermented sausage samples (S_2) contain *L.acidohpilus* (7 log CFU/ g) + *B.longum* 10^7 CFU /g had lowest value among all samples may be due to *B.longum* bacteria had low fermentation effect comparing to effect of *L.acidohpilus* bacteria, while fermented sausage samples (S_3) contain

L.acidohpilus+ allyl isocyothinat oil 500 ppm had highest value among all samples may be due to higher fermentation effect of *L.acidohpilus* comparing to *L.acidohpilus* and antibacterial effect of allyl isocyothinat oil which has spicy odor and flavor. From above mentioned results it is clear that all the samples are physically accepted but has different scores vary from like extremely in fermented sausage samples (S_3) contain *L.acidohpilus* (7 log CFU/ g) + allyl isocyothinat oil 500 ppm , like very much in fermented sausage samples (S_4) contain *L.acidohpilus*(7 log CFU/ g) + *B.longum* + allyl isocyothinat oil 500 ppm, like moderately in fermented sausage (S_1) samples contain *L.acidohpilus* and like slightly in fermented sausage samples (S_2) contain *L.acidohpilus* + *B.longum*.

Organoleptic examination revealed that fermented sausages contain 500 ppm AITC only were the most acceptable sausages as assessed by the sensory panels, and these sausages could be marketed as a safe specialty product such as a hot sausages.

From the work presented, there is a clear indication that both *Bifidobacterium longum* and AITC have strong bactericidal effects against *Salmonella typhimurium* in fermented sausage. The results listed in fig.(4) declared that The initial count of *Salmonella typhimurium* decreased during fermentation period of five days in all fermented sausage samples, but the higher reduction effect was in sausage (S4) contain both *Bifidobacterium longum* and AITC as *Salmonella* count were less than 100CFU/g, while the second reduction effect was obtained from fermented sausage (S3) contain AITC as *Salmonella* count was reduced to 2 log CFU/g, followed by fermented sausages (S2) contain *Bifidobacterium longum* only as the count reduced from 6.3 log CFU/g to 3 log CFU/g and fermented sausage (S1) with no additives count reduced to 4.3 log CFU/g. After dryness period (12 days of processing) *Salmonella* not obtained from fermented sausages (S4) contain both *Bifidobacterium longum* and 500 ppm AITC even after incubation at 37◦C for 24hrs on tryptic soya broth. AITC was reported to affect β galactosidase activity and induce cellular metabolic leakage, thereby inhibiting gram-negative bacteria **(Lim *et al.*, 1997).** Other mechanisms such as bactericidal action by disrupting disulphide bonds and affecting protein structures **(Kawakishi and Kaneko, 1987)** or the respiration of cells (**Kojima and Ogawa, 1971**), were proposed but the complete mechanism of action of AITC is still unknown. **Rhee *et al.*, 2003** found that mustard flour eliminate 6 logCFU/g salmonella to undetectable level when mustard samples stored at 5°C after one day.

However fermented sausages with AITC only, fermented sausages with *Bifidobacterium longum* only and that with no additives count reduced to 1.2, 2.9 and 4 log CFU/g respectively. After 7 days of storage *Salmonella* not obtained from fermented sausages contain AITC only even after incubation at 37°C for 24hrs on tryptic soya broth, while fermented sausages contain *Bifidobacterium longum* only and fermented sausage with no additives count reduced but the reduction was more higher in fermented sausage contain *Bifidobacterium longum,* this attributed to higher acid production of *Bifidobacterium longum* than do Lactobacillus acidophilus alone.

Shan *et al.*, 2001 showed that probiotic bacteria have great antimicrobial properties due to their production of organic acids, hydrogen peroxides, bactericides and bactericides like substances. Lactic acids and acetic acids account more than 90% of the produced acids from probiotic bacteria. After 47 days of processing Salmonella not obtained either from fermented sausage contain *Bifidobacterium longum* nor fermented sausages with no additives. The best reduction effects were obtained from fermented sausages contain AITC and *Bifidobacterium longum* followed by fermented sausages with AITC only then fermented sausages with *Bifidobacterium longum* only and fermented sausages with no additives. These results attributed to high acid production of probiotic and antibacterial effect of AITC. **Marshal and Tamime, 1997** reported that lactic acid bacteria including *Bifidobacterium* spp could produce bacteriocines which are protein in nature and have antibacterial effect against gram-negative bacteria. **Murad *et al.*, 2000** stated that bactericidal action of *Bifidobacterium longum* may be attributed to the production of some kinds of antimicrobial agents were relatively stable at acid production in addition to their pH reducing effect which inhibits food borne pathogens.

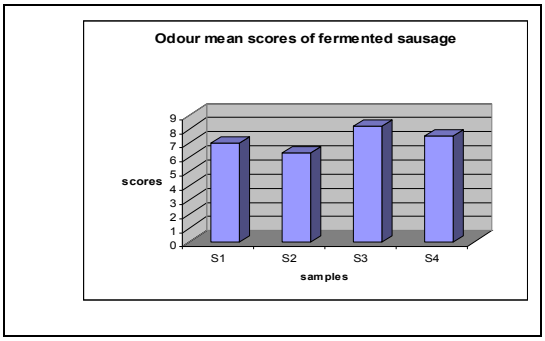

Fig (1) : Odor mean scores of fermented sausage
S1: fermented sausage Samples.
S2: fermented sausage Samples + *B. longum.*
S3: fermented sausage Samples + AITC.
S4: fermented sausage Samples + *B. longum* + AITC.

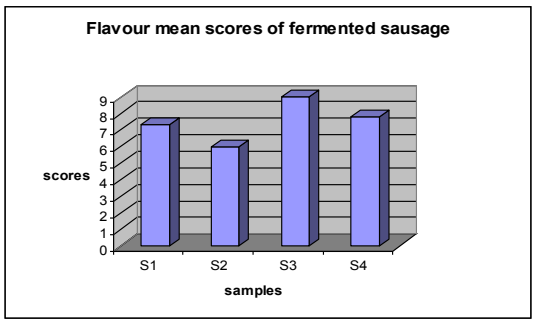

Fig (2): Flavor… mean scores of fermented sausage
S1: fermented sausage Samples.
S2: fermented sausage Samples + *B.longum*.
S3: fermented sausage Samples + AITC.
S4: fermented sausage Samples + *B.longum* + AITC.

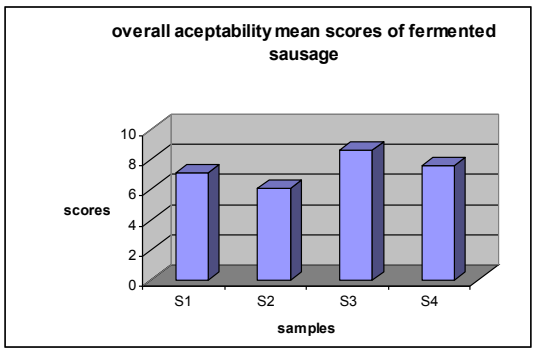

Fig (3): Overall acceptability mean scores of fermented sausage
S1: fermented sausage Samples.
S2: fermented sausage Samples + *B.longum*.
S3: fermented sausage Samples + AITC.
S4: fermented sausage Samples + *B.longum* + AITC.

Table (1): Viability of *Salmonella typhimurium* in fermented sausage:

	F.S	F.S+Bif	F.S+AITC	F.S+AITC+Bif
Zero	$6.3^{a\ I}$	$6.3^{a\ I}$	$6.3^{a\ I}$	$6.3^{a\ I}$
Fermentation (5days)	$4.2^{a\ II}$	$4^{b\ II}$	$2.1^{cb\ II}$	$0.9^{db\ II}$
Drying (7 days)	$4.1^{a\ II}_{III}$	$2.8^{b\ II\ III}$	$1.2^{cb\ II\ III}$	$0^{db\ II\ III}*$
Storage (19 days of processing)	$3.3^{a\ II}_{IV}$	$2.5^{b\ II\ IV}$	$0^{cb\ II\ IV}*$	$0.00^{db\ II\ IV}*$
26 days of processing	$3.1^{a\ II}_{V}$	$2.2^{b\ II\ V}$	$0.00^{cb\ II\ V}*$	$0.00^{db\ II\ V}*$
33 days of processing	$2.4^{a\ II}_{VI}$	$2.1^{b\ II\ VI}$	$0.00^{c\ II\ VI}*$	$0.00^{dc\ II\ VI}*$
40 day of processing	$2.1^{a\ II}_{VII}$	$1.6^{b\ II\ VII}$	$0.00^{cb\ II}_{VII}*$	$0.00^{db\ II\ VII}*$
47 day of processing	$0.00^{V}_{II\ III}*$	$0.00^{II}_{III}*$	$0.00^{II\ III}*$	$0.00^{II\ III}*$

No significant difference (P<0.05) between cells contain same letter in the same row.
No significant difference (P <0.05) between cells contain same Roman letter in the same column.
* After incubation at 37◦C for 24hrs on tryptic soya broth.

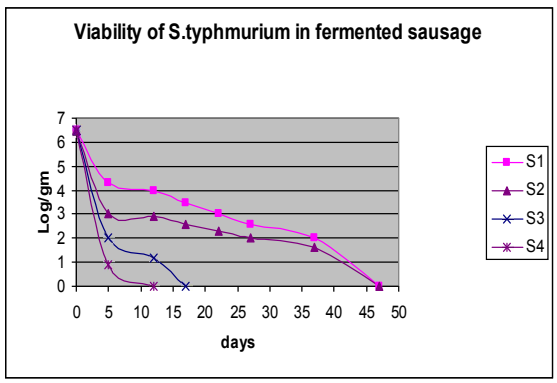

Fig (4): Viability of S.typhimurium in fermented sausage
S1: fermented sausage Samples.
S2: fermented sausage Samples + *B.longum*.
S3: fermented sausage Samples + AITC.
S4: fermented sausage Samples + *B.longum* + AITC.

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