Isolation and Determination of the Major Principle or Causative Agent behind the 2016 Published Breakthrough Discovery of Dr. M.S. Reddy’s “Multiple Mixed Strain Probiotic Therapy”, in Successfully Treating the Lethal Hospital Acquired Infections Due to *Clostridium difficile* (C. diff) and Methicillin Resistant Staphylococcus aureus (MRSA)

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ABSTRACT

A multiple mixed strain Probiotic culture was compounded using several naturally antibiotic resistant beneficial microorganisms belonging to different genera and species, along with their bacteriocins and growth end products or metabolites. Prior to mixing several individually grown single strains of Probiotics, compatibility studies were conducted to eliminate the strain dominance in the mixed cultures using direct differential plating techniques and strain specific bacteriophages, using the procedures outlined in our earlier publication. The multiple mixed strain Probiotics thus prepared were studied to see whether Probiotic bacteria by themselves or their bacteriocins and other end products of growth (without the live Probiotics) or the combination of live Probiotics and their bacteriocins and other growth end products, were responsible to inhibit the growth and proliferation of the lethal hospital acquired infections i.e., *Clostridium difficile* (C. diff) and Methicillin Resistant Staphylococcus aureus (MRSA). In addition, several procedures of preparing the functional mixed strain Probiotics, using liquid nitrogen freezing and freeze drying (lyophilization) were evaluated, to come up with the best procedure suitable to maximize their subsequent inhibitory effect on C. diff and MRSA, both under the laboratory conditions and in the practical clinical hospital conditions. Community based clinical trials were conducted to check the effect of multiple mixed strain Probiotics with and without their bacteriocins and their other growth end products, to cure the lethal *C. diff* infection under practical hospital conditions. The results revealed and confirmed that our 2016 novel discovery is still the best way to prepare and administer the multiple mixed strain Probiotics to treat the lethal hospital acquired infections. Also this study reconfirmed that the liquid nitrogen freezing is far superior to freeze drying or lyophilization to preserve the maximum efficiency of multiple mixed strain Probiotics. In conclusion, it proved that even the concentrated high bacterial cell number multiple mixed strain Probiotics (without their bacteriocins and other growth metabolites) are not as effective compared to the multiple mixed strain Probiotics frozen using liquid nitrogen, along with their bacteriocins and growth metabolites, to cure the lethal *C. diff* infection under practical clinical hospital conditions.

KEYWORDS: Probiotics; bacteriocins; MRSA; C. diff; multiple mixed strain Probiotic therapy; Multiple mixed strain Probiotics; Nosocomial infections; Hospital acquired infections; Natural antibiotic resistant Probiotics; Propionibacterium; lactic acid producing Probiotic bacteria.

Introduction

Over 6,000,000 people gets affected annually with the hospital acquired infections due to "*Clostridium difficile* (C. diff) and Methicillin resistant staphylococcus aureus (MRSA)" – the superbug. This statistic is according to the cases recorded and notified. In reality, it could be over 12,000,000 people, since some of the countries and hospitals do not keep the accurate records or reporting. On the record, a minimum of over 100,000 of those people who contact these secondary nosocomial infections die annually. Off the record, it can be over
1,000,000 and nobody knows the truth, since an accurate record keeping is not maintained in the hospitals. It is rather unfortunate that these patients were admitted for aspecific ailment and they end up dying due to completely unrelated nosocomial infections. These hospital acquired infections are due to the dominant multiple antibiotic resistant pathogenic bacteria, thus making it very difficult to treat them successfully. Talking about the strain dominance, it is interesting to know that in a newly born baby the majority of the gastrointestinal flora are Probiotics, especially lactic acid producing bacteria such as *Lactobacillus acidophilus* and several species of *Bifidobacterium*. It is because the baby is not exposed to all other bacterial species other than the mother’s vaginal flora, which are predominantly Probiotics comprising *Lactobacillus acidophilus*, *Bifidobacterium* species etc. As human being ages, the composition of the gastrointestinal flora changes depending upon the food habits, stress, the environment, life styles, diseases, and unscrupulous usage of drugs used (including but not limited to several antibiotics) to treat the diseases. Finally, in the old age, the number of Probiotics in the gastrointestinal tract decrease significantly in comparison to other microflora. This can be due to life styles, lack of resistance to fight pathogens, discrepancy in the immune modulation, unscrupulous use of drugs or due to unknown physiological changes associated with the old age, such as decreased peristalsis of the intestinal tract, decreased production of digestive enzymes and gastric acids, bed ridden situation leading to constipation etc. Consequently, it is extremely important to maintain the proper Probiotic bacterial population in the gastrointestinal tract as people age, to maintain the gastrointestinal eco flora or microbiota.

The gastric hydrochloric acid production decreases with age due to reduction of estrogen and testosterone hormones (Reddy and Reddy, 2011). It leads to not only decreased absorption of the essential minerals (which are catalysts to the action of digestive, cellular as well as intracellular enzymes) but also a significant change in the microbial composition of the gastrointestinal flora. Consequently, due to reduction of gastric acids, bile salts, and gastric enzymes, the tendency for the domination of non-beneficial microorganisms and pathogens will be significantly higher, in comparison to beneficial Probiotic bacteria, in the old age.

Irrespective of age, the unscrupulous use of antibiotics can also create the undesirable imbalance of the gastrointestinal microflora, specifically in the favor of multiple antibiotic resistant pathogenic bacteria such as *C. diff*, *MRSA* and other hospital acquired infections. The immune compromised individuals are especially susceptible for these infections due to dominant pathogenic microflora in the gastrointestinal tract. To make the situation worse, only limited number of available and existing antibiotics are effective against these nosocomial infections, making the therapeutic treatment efficiency as a chance rather than a choice. In other words, antibiotic treatment modality is a hit and miss proposition to cure the hospital acquired or associated infections. In this context, it is worthwhile to mention that as early as over 100 years ago, to be precise in 1907, Nobel Laureate Dr. Metchnikoff pointed out that the longevity of human being is dictated by the composition of the gastrointestinal microflora. He has also pointed out that certain lactic acid bacteria can prevent some intestinal ailments (Metchnikoff 1907). However not much attention has been paid because of the lack of evidence and also due to lack of extensive microbiological knowledge.

After the discovery and introduction of the first antibiotic penicillin in mid-1940’s, the entire medical community was in the favour of using antibiotics. Surprisingly the term Probiotic was not coined until middle or later part of the 20th century. The Probiotic concept was limited to very few bacteria such as *Lactobacillus acidophilus* and few species of *Bifidobacterium*. Later Fuller has refined the Probiotics as live microbial supplements that bestow beneficial effects on the host by improving the intestinal microbial balance (Fuller, 1989). It opened up the door for the scientific community worldwide to look into various beneficial microorganisms besides L. acidophilus and species or Bifidobacterium. If you analyze it, some of these Probiotics play a significant role in the human gastrointestinal tract. If the beneficial bacterial numbers significantly decrease in proportion to the non-beneficial pathogenic bacteria, the dominant pathogenic bacteria will cause disease, which is manifested by the clinical symptoms. One such situation is the multiple antibiotic resistant pathogenic bacteria encountered in the hospitals. This is the reason why the hospital acquired or nosocomial infections are of major concern in this day and age.

Earlier, we have outlined in detail the genesis and biochemical and microbiological characteristics of the causative agents of these hospital acquired disease causing lethal pathogenic bacteria i.e. *C. diff*, *MRSA*. We have also disclosed for the first time in the world, our major breakthrough discovery of successfully preventing or treating these hospital associated infections in a detailed article titled “Development of Multiple Mixed Strain Probiotics for “Probiotic Therapy” under clinical conditions, to prevent or cure the deadly hospital acquired infections due to *C. diff* and *MRSA*, published in the prestigious International Journal of Pharmaceutical Sciences and Nano Technology” volume 9. Issue 3. May-June 2016 (Reddy and Reddy, 2016). It is evident from this article that it took over 47 year’s worth of studious basic research by Dr. M.S. Reddy to arrive at the novel discovery to prevent or cure the hospital acquired infections using all natural live beneficial biological agents. This discovery took longtime and extensively laborious due to continuous genetic changes of the pathogens in response to the antibiotics. The discovery was based on the fact that the multiple antibiotic resistant hospital associated or acquired pathogenic bacterial infections can only be cured using the specific all natural antibiotic and/or antimicrobial agents resistant
multiple mixed strain Probiotics belonging to different genera and species as a therapeutic aide.

There are over 500 species of bacteria which harbour in the human gastrointestinal tract. These organisms are a mixture of facultative anaerobes, aerobes, microaerophilis and strict anaerobes. To date, nobody understands fully the exact and total composition and significance of the individual species and their physiological contribution in the human gastrointestinal tract. But the true fact is that human being will not survive if all of the intestinal microflora or microbiota are killed or eliminated. In this connection, it is worthwhile for the reader to review the research pertaining to the effect of several pharmaceutical antibiotics and antimicrobial agents on some of the human beneficial gastrointestinal flora (Reddy and Reinbold, 1972; Reinbold and Reddy, 1974). When a physician administers the course of antibiotics, to treat an ailment, indirectly such antibiotics are inhibiting not only the causative pathogenic bacteria but also the other nonpathogenic beneficial bacteria, thus creating an unnatural and dangerous strain dominance in the human gastro intestinal tract by the antibiotic resistant bacteria.

In reality, the pathogenic bacterial strain dominance was created due to elimination of the competing nonpathogenic and beneficial bacteria in the GI tract. Thus the multiple antibiotic resistant hospital acquired pathogenic microorganisms will dominate the GI flora to cause disease and ultimately, if not controlled, will kill the host. Earlier we have outlined various mechanisms by which the antibiotic resistance is developed by various pathogenic microorganisms (Reddy et al., 1973; Reddy and Reddy, 2016). Our discovery is novel in that we were able to select through elaborate screening some of the beneficial Probiotic bacteria belonging to various genera and species which are naturally resistant to several antibiotics and antimicrobial agents, thus can outcompete and inhibit the pathogens responsible for nosocomial infections, even when administered along with specific antibiotics (Reddy and Reddy, 2016). We have also discovered that several species of the beneficial Propionibacterium are naturally resistant to sulfonamides as a group (Reinbold and Reddy, 1972). We have also determined, using the radio isotope marked sulfadiazine, this natural resistance in Propionibacterium was due to lack of membrane transport system for sulfonamides (Reddy et al., 1973). Similarly, we have discovered that several other Probiotic beneficial microorganisms were also naturally resistant to some antibiotics (Reinbold and Reddy, 1974).

The novelty of our discovery was the use of the selective naturally antibiotic and/or antimicrobial agent resistant beneficial Probiotics to outcompete the antibiotic resistant hospital acquired infections due to C. diff and the MRSA (the superbug). The other novelty of discovery is that the naturally antibiotic resistant multiple mixed strain Probiotics can inhibit the C. diff and MRSA organisms even in the presence or absence of antibiotics such as vancomycin, bacitracin and sulfa-methoxazole, without altering the ecology of the microbiota of the human gastrointestinal tract. This is the first novel discovery in the world and to date nobody has ever attempted to develop such a well-researched and scientifically based biological system to cure the antibiotic resistant nosocomial infections using the pretested naturally selective antibiotic and antimicrobial agent(s) resistant multiple mixed strain Probiotics belonging to different genera and species as “therapeutic agents.”

The other novelty of the discovery is the importance of the procedure of preparing the naturally antibiotic resistant multiple mixed strain Probiotic cultures as therapeutic agents to efficiently cure the hospital acquired infections. Reddy et al. has determined that the beneficial mixed lactic cultures (Probiotics) are better preserved using liquid nitrogen freezing compared to the lyophilization in term of maintaining the component balance when subjected to preservation procedures (Reddy et al., 1971). The liquid nitrogen instantly freezes the cultures thus eliminating the membrane damage to the Probiotic bacterial cells. Unfortunately, for the sake of convenience, physicians prefer freeze dried (single strain) powdered cultures. They are easy to transport and convenient to administer. However, earlier while running the community based clinical trials, several physicians reported that freeze dried Probiotics are ineffective in curing the hospital associated infections (Reddy and Reddy, 2016). Our serendipitous and novel discovery of the use of multiple mixed strain Probiotic therapy using liquid nitrogen frozen cultures was very effective in preventing or curing the hospital acquired infections (Reddy and Reddy, 2016).

The current investigation is undertaken to study the following:

1. The effect of liquid nitrogen freezing vs. lyophilization of the multiple mixed strain Probiotic cultures and their effect on retarding the growth of C. diff and MRSA;
2. The effect of liquid nitrogen frozen concentrated multiple mixed strain Probiotic cultures (5 times concentrated) with no natural Probiotic produced bacteriocins and other metabolic end products vs. the less concentrated (Probiotic population) liquid nitrogen frozen multiple mixed strain Probiotics along with their natural Probiotic produced bacteriocins and other metabolic end products, on inhibiting the growth of C. diff and MRSA;
3. To study the effect of the liquid nitrogen frozen Probiotic cultures along with their bacteriocins and other growth end products vs. lyophilized multiple mixed strain Probiotic cultures along with their bacteriocins and other growth end products, to cure the C. diff infection in the hospital atmosphere as part of the community based clinical trial;
4. To study the effect of combining the multiple mixed strain Probiotic therapy along with the antibiotic therapy in curing the C. diff infection in the hospital environment;
5. To identify the major biological principle behind the success of our discovery of using the liquid nitrogen frozen multiple mixed strain Probiotics, in preventing or curing the hospital associated infections.

More specifically to answer the following pinpointing questions: Does multiple mixed strain Probiotic concentrated culture alone without their end products of growth and bacteriocins can cure the hospital acquired infections? Is it required or essential to have not only the live Probiotic bacterial cultures but also their end products of growth including bacteriocins to cure the nosocomial infections? The results of this investigation should reveal the main principle responsible for the success of Dr. M.S. Reddy’s discovery of the use of liquid nitrogen frozen multiple mixed strain Probiotic cultures as therapeutic agents, to prevent or cure the lethal hospital acquired infections.

Thus, our current approach in this investigation to include the all-natural specific antibiotic resistant, nonpathogenic beneficial Probiotic bacteria belonging to various genera and species as biological agents, used in the discovery of Reddy and Reddy (Reddy and Reddy, 2016), to compound the multiple mixed strain Probiotic culture which can successfully and predictably outcompete the growth and proliferation of the C. diff, MRSA. Reddy and Reddy have outlined the methodology and preparation of such multiple mixed strain cultures to prevent or cure these nosocomial infections (Reddy and Reddy, 2016). Dr. M.S. Reddy’s discovery was practical and outstanding in that after he announced it in the form of written publication in the major journals (Reddy and Reddy, 2016) and presentations at the major physicians conventions (Reddy and Reddy, 2008), several hospitals around the world started to practice the technique of using multiple mixed strain Probiotics (although not completely refined) to cure these deadly nosocomial infections, with or without using antibiotics as a primary or complementary therapy. Although the discovery of using multiple mixed strain Probiotic therapy was novel and very effective in treating these diseases, we could not pinpoint the exact principle behind curing of these infections. The following questions have never been answered before: Does bacteriocins alone produced by multiple mixed strain Probiotics responsible for inhibiting C. diff and MRSA infections? Or does live multiple mixed strain Probiotics which produced the bacteriocins responsible? Do we need both the live multiple mixed strain Probiotics and their growth end products including bacteriocins to cure the nosocomial infections? This investigation is undertaken to unveil the main principle behind the success of Dr. M.S. Reddy’s breakthrough serendipitous discovery. This will help in the immediate future to develop successful and relatively simpler treatment modalities (to put this discovery in practice around the world) with 100 percent success to curb these deadly lethal hospital acquired infections, which are growing rapidly and killing several innocent people in the world.

### Materials and Methods

Several pretested (for antibiotic resistance) Probiotic strains utilized in the novel discovery of multiple mixed strain Probiotic cultures to prevent or treat the hospital acquired infections (Probiotic Therapy) outlined by Reddy and Reddy (Reddy and Reddy, 2016) were used in the preparation of the current multiple mixed strain Probiotic culture. The microbiological composition of the multiple mixed strain culture (prepared and tested in this investigation) is presented in Table 1.

#### TABLE 1

The microbiological composition of the multiple mixed strain probiotic culture.

<table>
<thead>
<tr>
<th>No.</th>
<th>Probiotic strains belonging to different genera and species included in the current multiple mixed strain Probiotic culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lactobacillus acidophilus</td>
</tr>
<tr>
<td>2</td>
<td>Bifidobacterium bifidus</td>
</tr>
<tr>
<td>3</td>
<td>Leuconostoc dextranicum</td>
</tr>
<tr>
<td>4</td>
<td>Leuconostoc citrovorum</td>
</tr>
<tr>
<td>5</td>
<td>Lactobacillus sporogenes</td>
</tr>
<tr>
<td>6</td>
<td>Propionibacterium shermanii</td>
</tr>
<tr>
<td>7</td>
<td>Propionibacterium zeae</td>
</tr>
<tr>
<td>8</td>
<td>Streptococcus lactis</td>
</tr>
<tr>
<td>9</td>
<td>Streptococcus cremoris</td>
</tr>
<tr>
<td>10</td>
<td>Streptococcus diacetylactis</td>
</tr>
<tr>
<td>11</td>
<td>Streptococcus thermophilus</td>
</tr>
<tr>
<td>12</td>
<td>Lactobacillus bulgaricus</td>
</tr>
<tr>
<td>13</td>
<td>Suecchoromycos boulardii</td>
</tr>
<tr>
<td>14</td>
<td>Brevibacterium linens</td>
</tr>
<tr>
<td>15</td>
<td>Streptococcus durans</td>
</tr>
</tbody>
</table>

To eliminate the confusion of the nomenclature of the genera and species of some of the Probiotics, we have elected to give the old and new nomenclature as outlined in the Bergeys manual of the determinative bacteriology and other publications, since they are used interchangeably in several journal articles. They are as follows: Streptococcus lactis (old) - Lactococcus lactis var lactis (new); Streptococcus cremoris (old) - Lactococcus lactis var cremoris (new); Streptococcus diacetylactis (old) - Lactococcus lactis var lactis subsp. diacetylactis (new); Streptococcus thermophilus (old) - Streptococcus salivarius subsp. thermophilus (new); Lactobacillus bulgaricus (old) - Lactobacillus delbruekii var bulgaricus (new); Streptococcus durans (old) - Enterococcus faecium (new); Leuconostoc citrovorum (old) - Leuconostoc mesenteroides subsp. cremoris (new); Leuconostoc dextranicum (old) - Leuconostoc mesenteroides subsp. dextranicum (new); Lactobacillus sporogenes (old) - Bacillus coagulans (new) - clarified according to Vecchi and Drago (Vecchi and Drago, 2006).

Even in this article, we have used the old and new nomenclature interchangeably because of the continuum of this research for over 47 years. We are proud to announce that we were the first people in the world to introduce the beneficial species of Propionibacterium as Probiotics. We were also the first people in the world to introduce Probiotics as part of the functional therapeutic agents along with the complementary drugs (as part of the drug modality) to enhance the effect of drug activities.
to cure the diseases at a much faster rate, yet with no serious side effects. Thus a patent has been granted signifying that the invention is new and novel in the medical and nutraceutical field in the world (Reddy et al., 2000).

In our earlier investigation, we have discovered that certain Probiotics are very effective as preventive agents and certain other Probiotics are good as treatment agents based on their antibiotic resistance and sensitivities. Since we are trying to investigate to pinpoint the major principle or ingredient behind the successful discovery of the use of multiple mixed strain Probiotic cultures to cure the *C. diff* and *MRSA* infections, we have elected to use various pretested Probiotic strains (belonging to different genera and species), as outlined in the discovery of Reddy and Reddy (Reddy and Reddy, 2016). Most of the Probiotic bacterial cultures used in this study are naturally resistant to several antibiotics and sulfonamides. One can argue that such antibiotic or antimicrobial resistance can be due to plasmids or extra chromosomal genes, thus casting a doubt stating that such antibiotic resistance of Probiotics can be transferred to the other pathogens. To the best of our knowledge, the natural antibiotic and antimicrobial resistance encoding genes in the Probiotic cultures (used in this investigation) are chromosomal rather than extra chromosomal or plasmids, since these organisms have been used in the food industry for over a century without any problems and thus the Food and Drug Administration has granted them GRAS (generally regarded as safe) status to be used safely in the manufacture of dairy and other food products (Code of Federal Regulations of Food and Drug, 2001). However, to prove that the Probiotics used in this investigation are naturally resistant to several antibiotics and antimicrobial agents, and furthermore to prove such resistance was not coded by the plasmids, we have conducted several intricate laboratory experiments.

Generally, if an organism has a specific character, which is coded by the extra chromosomal gene or plasmid, such a character is not permanent, as opposed to if the gene is located in the chromosome. The chromosomal genes are permanent. To prove this, we have grown the single strain Probiotic cultures about 5 to 7 degrees over and above their maximum threshold growth temperature (beyond which the culture will not grow). Such cultures were transferred and grown for serially 5 more times at the elevated growth temperatures. After the fifth transfer and growth they were tested for their antibiotic resistance. If both the high temperature grown, and the optimum temperature grown culture (control) exhibit no signs of inhibition by a specific antibiotic or antimicrobial agent (which was proven to be non-inhibitory to the test culture), it is an indication that the gene responsible for the natural antibiotic resistance resides in the chromosome, as opposed to existing as a plasmid. As an illustration, we have grown the *lactococcus lactis var lactis* strains (proven to have lactose utilizing gene residing autonomously as plasmid rather than chromosomal) both at high temperature and optimum temperature and checked for the presence or absence of the lactose positive character using the differential agar of Reddy et al. (Reddy et al., 1969) with lactose as a sole source of carbohydrate (without any arginine or milk or glucose) with bromocresol purple as an indicator dye. The colonies of the lactose positive cultures will be large on surface plated differential agar and turn distinctly yellow due to acid production. Whereas, their lactose negative counterparts will be small and will not develop yellow color colonies, due to lack of plasmids coding for enzymes required for the lactose utilization. Our test, using this methodology confirmed that the lactose positive character in this particular bacterial strain is not chromosomal but it is extrachromosomal or plasmid controlled, which is proven due to loss of lactose positive character upon high temperature incubation. Using the similar test protocol, by changing the substrate in the differential agar to glucose (without any arginine or milk or lactose) we have confirmed both the higher temperature grown and optimum temperature grown *lactococcus lactis var lactis* strain grew on differential agar with distinct yellow colonies, indicating that the bacterial genes responsible for the glucose utilization reside on chromosome rather than being extrachromosomal or reside as plasmids. This is qualitative test and it is very easy to test the Probiotic cultures *in vitro*. When an organism is subjected to growth continuously at very high temperatures (significantly over and above their maximum growth temperature) they tend to loose their plasmids, upon several transfers.

The following Probiotic cultures and antibiotics were tested to check whether the gene conferring the antibiotic resistance reside on chromosome or autonomous plasmid. Selective strains of *Streptococcus lactis*, *Streptococcus diacetylactis* and *Streptococcus durans* cultures subjected to growth at high and optimum temperatures (transferred successively 5 times) were checked for lack of inhibition by polymyxin B, at 50 units/disc. Similarly, vancomycin at a concentration of 30mcg/disc was checked on strains of *Leuconoctoc dextranicum*. Effect of antibiotic colistin at 2mcg/disc was checked against the strains of *Streptococcus thermophilus*. The strains of *Propionibacterium* were checked for antibiotic resistance using gantricin at 50mcg/dis, using the procedure outlined by Reddy et al. (Reddy et al., 1973). The procedure of Reinbold and Reddy (Reinbold and Reddy, 1974) was used to check for the antibiotic resistance or sensitivity of other Probiotics tested. The results of the aforementioned microbiological tests proved that the Probiotic bacterial resistance (used in this investigation) towards some antibiotics and antimicrobial agents are due to chromosomal genes rather than the plasmids, because both the control as well as the high temperature grown Probiotics exhibited similar resistance patterns. It goes to prove that some of the single strains belonging to different genera and species used in our multiple mixed strain Probiotics are safe to use clinically to inhibit the *C. diff* and *MRSA*, without having to worry about transfer of such antibiotic resistance to other pathogenic bacteria.
The multiple mixed strain Probiotic cultures were grown individually using the procedure outlined by Reddy and Reddy (Reddy and Reddy, 2016). However, in this investigation, we have elected to grow the individual cultures longer (two additional hours after early stationary phase of growth) to slightly maximize the production of bacteriocins and other growth metabolites. The fully grown individual cultures were then mixed in equal proportions. The mixed culture was chilled to prevent further growth of the strains in the mixed strain culture to eliminate the unexpected strain dominance, since the earlier investigators have determined that strain dominance exists even among the closely related Probiotic bacteria when they are grown together (Reddy et al., 1971-1972). This mixed culture was divided into six fractions. Each fraction was treated differently and details are as follows:

**Fraction 1**: The mixed culture as is (not centrifuged) frozen using liquid nitrogen.

**Fraction 2**: The mixed culture as is (not centrifuged) but freeze dried.

**Fraction 3**: The mixed culture was centrifuged (the supernatant separated) and the concentrated culture was diluted to the same volume as the uncentrifuged culture, and then centrifuged again to obtain bacterial cells only, without their bacteriocins and other metabolites. Finally, the concentrated culture was once again diluted to the original volume using chloride free sterile distilled water and then frozen using liquid nitrogen.

**Fraction 4**: Prepared using the same procedure as outlined in fraction 3, except at the final step, it was freeze dried as opposed to liquid nitrogen freezing.

**Fraction 5**: The liquid mixed culture was centrifuged and the concentrated culture was diluted in sterile distilled water to the original volume and then centrifuged to concentrate the cells. The concentrated cells were then frozen using liquid nitrogen.

**Fraction 6**: Prepared in same fashion as above except the final concentrated culture is lyophilized or freeze dried as opposed to liquid nitrogen freezing in fraction 5.

All of the above preparations were checked in the laboratory (in vitro) for the degree of inhibition on *C. diff* and *MRSA* culture, using the procedure outlined by Reddy and Reddy (Reddy and Reddy, 2016). The results are presented in table 2 and table 3.

### TABLE 2

<table>
<thead>
<tr>
<th>Probiotic preparation identified as fractions</th>
<th>Degree of inhibition on <em>C. diff</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>++++</td>
</tr>
<tr>
<td>Fraction 2</td>
<td>++</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>++</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>+</td>
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<tr>
<td>Fraction 5</td>
<td>++</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>+</td>
</tr>
</tbody>
</table>

The effect of various methods of culture preparation and preservation (fractions 1 to 6) upon the ability of mixed strain Probiotic culture on inhibition of *Clostridium difficile (C. difficile)* using the disc assay.

### TABLE 3

<table>
<thead>
<tr>
<th>Probiotic preparation identified as fractions</th>
<th>Degree of inhibition on <em>MRSA</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>++++</td>
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<tr>
<td>Fraction 2</td>
<td>++</td>
</tr>
<tr>
<td>Fraction 3</td>
<td>++</td>
</tr>
<tr>
<td>Fraction 4</td>
<td>+</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>++</td>
</tr>
<tr>
<td>Fraction 6</td>
<td>+</td>
</tr>
</tbody>
</table>

The community based clinical trials were conducted using the procedures outlined by Reddy and Reddy (Reddy and Reddy, 2016). However, the clinical trials conducted were limited to cure the *C. difficile* infection only. The details are presented under the following results and discussion section.

### Results and Discussion

The inhibition studies clearly proved that fraction 1 is more effective in inhibiting both the *C. difficile* and *MRSA* in vitro. Fraction 3 has exhibited 50% reduction in inhibition, indicating that bacteriocins and other culture metabolites are essential for 100% inhibition of the *C. difficile* and *MRSA*. The supernant obtained from fraction 3, was also tested to check for its ability to inhibit the growth of the *Clostridium difficile* and *Methicillin resistant staph aureus*. The supernant had a total concentration of 10,000 organisms per gram, which is considered a very low number of Probiotics compared to 10 billion organisms per gram in the culture without centrifugation. The results indicated that the supernatant with such low number of live Probiotic culture also exhibited inhibition of *C. difficile* and *MRSA* which is similar to the high concentration mixed strain of Probiotic culture itself (fraction 5) indicating that the end products of the Probiotic cultures (with even low number of Probiotics) have similar inhibitory effect compared to high concentration of mixed strain Probiotic cultures, without their bacteriocins and growth end products (fraction 5). This is an excellent discovery in that the administration of even the most concentrated Probiotic bacteria alone (without their bacteriocins and metabolites) cannot have significant effect in curing *C. difficile* and *MRSA* infection.

Also, the liquid nitrogen frozen culture along with their metabolites (fraction 1) is significantly better in inhibiting the *C. difficile* and *MRSA* compared to freeze dried Probiotic culture (fraction 2), indicating that freeze drying has detrimental effect on Probiotic cell membranes and their membrane bound enzymes. It reconfirmed the earlier physicians observation (while running the clinical trials) that freeze dried Probiotics were not at all effective in treating *C. difficile* or *MRSA* infection, compare to the liquid nitrogen frozen cultures (Reddy and Reddy, 2016). Even though fraction 5, has 5 times more mixed Probiotic bacterial cell population compare to fraction 1, yet the inhibition of *C. difficile* and
strains Probiotics to be 100% effective to inhibit the C. diff. It clearly proved that in order for the multiple mixed and growth end products exhibited maximum inhibition. bacterial cells-fraction 1) along with their bacteriocins mixed strain Probiotics (with high concentration of live hospital associated infections. However, the multiple growth end products of the multiple mixed strain products) indicating that the bacteriocins and other growth end products of the multiple mixed strain cultures. Surprisingly the inhibitory effect of the Probiotic supernatant of Fraction 3 (with 10,000 per ml Probiotic metabolites) are similar to non-filter sterilized bacteria-free supernatant (with bacteriocins and other specific and nonspecific metabolites etc.) using liquid nitrogen. The freeze dried preparations even with the culture metabolites, exhibited 50% less inhibitory effect than the liquid nitrogen frozen cultures.

Additionally, the supernatant from fraction 3 was filter sterilized to eliminate the total live Probiotic cultures, and checked for its ability to inhibit C. diff and MRSA strains. Surprisingly the inhibitory effect of the Probiotic bacteria free supernatant (with bacteriocins and other metabolites) are similar to non-filter sterilized supernatant of Fraction 3 (with 10,000 per ml Probiotic population along with their bacteriocins and growth end products) indicating that the bacteriocins and other growth end products of the multiple mixed strain Probiotics have a significant effect on inhibiting the hospital associated infections. However, the multiple mixed strain Probiotics (with high concentration of live bacterial cells-fraction 1) along with their bacteriocins and growth end products exhibited maximum inhibition. It clearly proved that in order for the multiple mixed strain Probiotics to be 100% effective to inhibit the C. diff and MRSA, it is imperative that their bacteriocins and other growth end products must be present along with the high concentration of live strains of Probiotics. This is a breakthrough discovery and this is the reason why Dr. M.S. Reddy's discovery of using multiple mixed strain Probiotic therapy was successful to prevent or cure the C. diff and MRSA infections in the hospital atmosphere, which was proven earlier by the community based clinical trials (Reddy and Reddy, 2016).

After running the laboratory tests in vitro, we have conducted the community based clinical trials in the hospital environment. The procedure used to conduct the clinical trials was same as outlined in the publication of Reddy and Reddy (Reddy and Reddy, 2016), except the study was limited to treating the C. diff infection only, using fraction 1 and 5 multiple mixed strains Probiotic cultures.

Practical community based clinical trials were conducted using multiple mixed strain Probiotic cultures (fraction 1 and 5), to arrive at the best way to prepare the multiple mixed strain Probiotic cultures, to treat the hospital acquired infections.

Since it is proven beyond any doubt (in in vitro studies), that the multiple mixed strain Probiotics with their inherently produced bacteriocins and other specific and nonspecific metabolites have significant inhibitory effect on the C. diff and MRSA organisms, we have elected to limit the clinical studies to fraction 1 and fraction 5 only. The only difference between fraction 1 and fraction 5 is that fraction 1 has lower Probiotic cell numbers and has all the bacteriocins and other growth end products of the multiple mixed strain Probiotics. Whereas, fraction 5 has five times more Probiotic bacterial cell population (compared to fraction 1) with no trace of bacteriocins and other culture growth end products. The multiple mixed strain Probiotic cultures, identified as fraction 1 and fraction 5, were frozen in liquid nitrogen in 330 gram metal cans and shipped in dry ice to the hospitals. In the hospital atmosphere, the physicians were asked to keep the cultures in freezers until used. The thawing and administering procedures were same as outlined in the paper published by Reddy and Reddy (Reddy and Reddy, 2016). The participating physicians were asked to give the multiple mixed strain Probiotics identified as fraction 1 and 5 for certain designated number of patients, who were confirmed infected with C. diff (while they were hospitalized). They were asked to observe for the cessation of C. diff infection symptoms, with conformation of absence of C. diff at the end of the treatment. They were also warned that, if patient does not show any symptoms of relief and the case is severe, they can give the antibiotics (bacitracin and vancomycin) along with the test Probiotic fraction, to eliminate further complications. However, they were warned that the antibiotics should be administered two hours prior to giving the multiple mixed strain Probiotics.

The results of the clinical trials, according to the physicians are listed in Table 4. The C. diff infection was completely cured in most of the cases (90 to 95%) using fraction 1 Probiotic preparation. In few acute life threatening cases (although it was not required) physicians gave antibiotics vancomycin and bacitracin, along with the fraction 1 (multiple mixed strain Probiotic culture) to cure the C. diff infection, to eliminate taking chances. Whereas, fraction 5 was not proven satisfactory and the symptoms of C. diff infection continued beyond 3 days. Thus, the participating physicians decided to give additional antibiotics along with fraction 5. According to all the clinical study participating physicians, fraction 1 is the best multiple mixed strain Probiotic which performed very well, even without the use of antibiotics. This was further proven by the significant reduction of C. diff bacterial count upon microbiological testing of the patient’s faeces, in addition to the cessation of the clinical symptoms.
TABLE 4
The effect of fraction 1 and fraction 5 of the mixed strain Probiotic cultures upon curing the C. diff infection in the community based clinical studies conducted in the hospital atmosphere, along with and without the use of antibiotics vancomycin and bacitracin.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Cessation of C. diff symptoms expressed in terms of percentage of cure in one week, upon using the multiple mixed strain Probiotic therapy only</th>
<th>Percentage cure of the C. diff infection by Probiotic fraction along with the use of antibiotics vancomycin and bacitracin in one week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction 1</td>
<td>90 to 95 percent*</td>
<td>100 percent</td>
</tr>
<tr>
<td>Fraction 5</td>
<td>&lt;10 percent**</td>
<td>50 percent</td>
</tr>
</tbody>
</table>

Footnote: *Fraction 1 totally cured C. diff infection without the use of antibiotics. In only 5% of the cases, the antibiotics were used along with Probiotics (fraction 1) for safety measure only. **Fraction 5 did not cure the C. diff infection and thus the antibiotics vancomycin and bacitracin were used after 3rd day along with fraction 5. The total cure was obtained only in 50 percent of cases in one week period.

It is very hard to run the formal clinical trials with these hospital associated infections. However, using the community based clinical trials we were able to conduct the study successfully. Since these hospitals associated infections are severe and life threatening to the hospitalized patients, at least some of the physicians and hospitals were interested in participating in these community based clinical trials. It has also been reported that several hospitals were closed both in USA and Europe because of the increased incidences of these uncontrollable hospital associated infections. Some of these virulent antibiotic resistant pathogenic bacteria are constantly mutating and thus making the treatment very difficult. Our discovery of using multiple mixed strain Probiotics together with their metabolites and bacteriocins (proven both in vitro and vivo) is the best way to prevent or treat these deadly hospital acquired (nosocomial) infections, which are affecting the lives of millions and millions of people in both the developed, developing, and underdeveloped countries throughout the world. If you analyze the practical clinical data, it is apparent that the growth end products of the multiple mixed strain Probiotics (along with Probiotics) have significantly greater effect than the Probiotics itself (with no bacteriocins and growth end products), even if the concentration of Probiotics are significantly higher. Apparently, when once C. diff infection is established, the Probiotic bacteriocins and other specific and nonspecific metabolites of the Probiotics start the initial inhibition process (primary) on C. diff and thus assist the live Probiotics to establish and continue their action (secondary). This is a significant discovery and proves that merely giving high concentration of Probiotics (especially freeze dried preparations), without their bacteriocins and other metabolites is not at all effective in treating the hospital acquired infections.

The participating physicians made a comment that the fraction 1 multiple mixed strain Probiotic frozen in liquid nitrogen, is by far the best preparation they have found to cure the C. diff infection. If you analyze the data, earlier (Reddy and Reddy, 2016) we have used only the antibiotic resistant Probiotic strains belonging to Leuconostoc dextranicum, Saccharomyces bouardii, Propionibacterium shermanii, Streptococcus durans and Lactobacillus sporogenes to treat the C. diff infection. In this study, in addition to the above strains, we have also included Lactobacillus acidophilus, Bifidobacterium bifidus, Leuconostoc citrovorum, Propionibacterium zeae, Streptococcus lactis, Streptococcus cremoris, Streptococcus diaylactis, Streptococcus thermophilus and Lactobacillus bulgaricus. Also, all these Probiotic cultures were incubated two hours longer than the previous methods reported earlier. The improved efficiency of this multiple mixed strain Probiotic culture could be due to the inclusion of additional Probiotic strains, thus having different spectrum of bacteriocins and/or more production of the amount of bacteriocins due to longer incubation of the Probiotic cultures. In any event, the C. diff infection was cured completely with the use of the current multiple mixed strain Probiotic culture with its bacteriocins and other specific and nonspecific inhibitory compounds, prepared by freezing in the liquid nitrogen.

Although, the above culture with bacteriocins and other metabolites totally cured the C. diff infection (in majority of the cases), physicians did not want to take risk in some severe cases and thus have administered antibiotics as a precautionary measure. The clinical study participating physicians came to a firm conclusion that the Probiotic cultures must have their bacteriocins and other metabolites to completely cure the C. diff infection. According to them it is the first breakthrough discovery, to treat the hospital acquired infections with accuracy and confidence in the world. They were also impressed by the fact that specific antibiotics can also be administered along with the multiple mixed strain Probiotics to treat the terminally ill patients. Also, the discovery is novel in that the mere high population of Probiotics (without their bacteriocins and metabolites) alone is not sufficient to cure the C. diff infection. Another novelty of this discovery is that the selection and blending of the strains in the multiple mixed strain Probiotics (inherent antibiotic resistance) is extremely important. In addition to the above, this discovery revealed that the method of growing the selected Probiotic strains belonging to different genera and species, and the method of preparing and preserving the mixed culture i.e. liquid nitrogen freezing, is extremely important. The use of popular procedure of concentrating the Probiotic cells without their bacteriocins and metabolites and then freeze drying them is ineffective and does not cure the C. diff infection. The future research should be concentrated on producing the maximum amount of bacteriocins, prior to liquid nitrogen freezing of the cultures, to make them still more efficient therapeutic agents.

Conclusions
This investigation confirmed the merit of Dr. M.S. Reddy’s breakthrough discovery of using multiple mixed...
strain “Probiotic therapy” to cure the C. diff infection in the hospital environment. Our laboratory tests further proved that the Probiotic cultures used in this investigation have to be grown and then mixed together (along with their bacteriocins and the growth end products) prior to freezing them using liquid nitrogen. Most of these Probiotic strains used in this discovery produce significant amount of bacteriocins and other metabolites. For example, some of the acid producing Probiotics produce copious amount of organic acids (lactic, acetic etc.), molecular hydrogen peroxide, and other nonspecific inhibitory compounds. The aforementioned inhibitory compounds in conjunction with the multiple bacteriocins, produced by the multiple mixed strain Probiotics, inhibit the pathogenic bacteria associated with the nosocomial infections, even at a Nano Molar concentration.

The strains of Propionibacterium used in the multiple mixed strain Probiotic cultures produce copious amounts of propionic, acetic and butyric acids (Vorobjeva et. al., 2008). These organisms are anaerobic and establish well in the gastrointestinal tract (Jan et. al., 2002; Vorobjeva et. al., 2008). In addition, these organisms produce probacteriocins which will be converted to bacteriocins upon partial hydrolysis by the proteolytic enzymes (Holo et. al., 2002). These bacteriocins produced by Propionibacterium are effective in retarding the pathogenic bacteria. Several beneficial Propionibacterium species exhibit both protective and reactivate activities due to production of intracellular protein identified as cysteine synthetase (Vorobjeva et. al., 2008). According to the basic researchers Warminiska-Radiko et. al., Propionibacterium species produce bifidogenic factors which stimulate the beneficial GI tract associated species of the Bifidobacterium (Warminiska-Radiko et. al., 2006). We have also included Streptococcus durans as part of the multiple mixed strain Probiotic because of the durability of this organism to survive and establish in the gastrointestinal tract. This particular strain has been proven to be non-pathogenic and has been used extensively in the US food industry as part of the starter culture to manufacture the dairy products.

The most important aspect of this discovery is that the liquid nitrogen frozen multiple mixed strain Probiotic cultures along with their bacteriocins and other metabolites had significant inhibitory effect on the pathogens C. diff and MRSA. When such a culture is washed and administered without its growth end products and bacteriocins, its inhibitory activity dropped by 50 percent. Even 5 times more concentrated bacterial cell population of the mixed strain Probiotic culture (without their bacteriocins and growth end products or metabolites) was not as effective as the significantly less bacterial cell population mixed Probiotic culture with its bacteriocins and the metabolites. It goes to prove that the bacteriocins and other prior growth end products of the multiple mixed strain Probiotics start the inhibition process (in vivo) of dominant multiple antibiotic resistant hospital associated infections. After this initial phase, the live Probiotic bacteria can establish themselves in the GI tract and continue their inhibitory effect on the C. diff and MRSA. This investigation also proved that the cell free extracts of the multiple mixed strain Probiotics have inhibitory effect on C. diff and MRSA. Neither the Probiotic bacterial cell free extracts with bacteriocins and other metabolites nor the bacterial cells of the multiple mixed strain Probiotic cultures (without the bacteriocins and metabolites) exhibited maximum inhibition on C. diff and MRSA, compared to the multiple mixed strain Probiotics with their intact bacteriocins and other metabolites. This investigation has proven beyond doubt and further strengthened and validated the significance of Dr. M.S. Reddy’s earlier discovery of using the multiple mixed strain Probiotics (along with their bacteriocins and other growth end products) to cure the C. diff and MRSA infections in the hospitals.

This investigation also proved that the method of preparing the multiple mixed strain Probiotics is of prime importance. There was 50% reduction in the inhibitory effect of the multiple mixed strain Probiotics between liquid nitrogen freezing and freeze drying, in that liquid nitrogen was far superior in preserving the activity of both the Probiotic bacterial cells as well as their bacteriocins and other metabolites. Up until now, the popular practice of preserving or preparing the concentrated single strain Probiotics is through lyophilization. There are two major drawbacks in this method of preparation i.e. 1. The bacterial cells are concentrated using centrifugation or filtration, which will remove most of the bacteriocins and other growth metabolites; 2. The concentrated cells (without their bacteriocins and metabolites) are then freeze dried, which is not as good as liquid nitrogen freezing to protect the viability and membrane stability of the Probiotic cultures. This investigation proved that lyophilized multiple mixed strain Probiotic cultures had exhibited least inhibitory effect on the C. diff and MRSA bacteria.

In this investigation, we have limited the clinical studies only to study the effect of liquid nitrogen frozen multiple mixed strain Probiotics with their bacteriocins and their growth end products vs. the liquid nitrogen frozen concentrated (5 times more bacterial population) multiple mixed strain Probiotics without their bacteriocins and their growth end products, on treating the C. diff infection in the hospital atmosphere. The community based clinical studies proved that even the most concentrated multiple mixed strain Probiotic cultures (without their bacteriocins and growth end products) were not very effective in curing C. diff infection. Whereas, the liquid nitrogen frozen multiple mixed strain culture (along with their bacteriocins and growth end products) cured the C. diff infection in less than a week. In some severe life threatening cases (<5%), physicians have elected to give the antibiotics vancomycin and bacitracin along with the multiple mixed strain Probiotic cultures to totally cure the infection. According to the physicians, although it was not required, they did not want to take chances and thus opted to administer antibiotics simultaneously. This further proved that our “Probiotic therapy” is
complementary with “antibiotic therapy” and thus can be used in acute life threatening cases.

The liquid nitrogen frozen concentrated multiple mixed strain Probiotics without their bacteriocins and the prior growth end products did not cure the *C. diff* infection in 90 percent of the cases. Thus physicians, when they did not notice any reduction of *C. diff* infection symptoms by the third day, they have elected to use the vancomycin and bacitracin along with the multiple mixed strain Probiotic cultures (without their bacteriocins and growth end products). According to them, using this approach, only 50% of the patients showed recovery in the first week. Thus they had to continue the combination treatment involving Probiotics and antibiotics until the patient is cured. They have all reported that antibiotic treatment alone using bacitracin and vancomycin is not at all a choice treatment to cure the *C. diff* infection. In fact, several patients could not be cured of *C. diff* infection with antibiotics, and ended up dying with the infection in hospitals.

The physicians came to a firm conclusion that the best treatment modality to cure the deadly hospital acquired *C. diff* infection is by using the discovery of Dr. M.S. Reddy (Reddy and Reddy, 2016) i.e. use of specific antibiotic resistant multiple mixed strain Probiotics along with their bacteriocins and growth end products or metabolites, frozen in the liquid nitrogen. Although we have limited these clinical studies to treat *C. diff* and *MRSA* infections (which are the major threats), this pioneering breakthrough discovery of Dr. M.S. Reddy (studiuously researched over a span of 47 years) can be extended to cure several other nosocomial infections and other gastrointestinal tract ailments such as Crohn’s disease, irritable bowel syndrome (IBS), intestinal diverticulosis, ulcerative colitis, celiac disease, irritable bowel disease (IBD), *H. Pylori* infections, and some colon cancers (Reddy and Reddy, 2007; 2008; 2015) etc. either as a primary therapy or as a complementary therapy.

In addition, this investigation points out the fact that the partial success of unaesthetic and not so safe fecal therapy to treat the terminal *C. diff* infection could be due to the bacteriocins and growth end products of the healthy donor fecal bacteria, rather than the solely undefined fecal bacteria themselves. This pioneering discovery of Dr. MS Reddy’s multiple mixed strain Probiotic therapy serves as a spring board to further the research into this beneficial Probiotic microbiological arena.

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