Analysis of the Comparative Occurrence of *Salmonella* Typhimurium and Its Monophasic Variant (4,[5],12:i:-) in Healthy and Clinically Ill Pigs in Northern Italy

Mario D'Incau (mario.dincau@izsler.it)
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
https://orcid.org/0000-0001-9918-2497
Cristian Salogni
Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna Bruno Ubertini
Stefano Giovannini
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
Jessica Ruggeri
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
Federico Scali
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
Matteo Tonni
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
Nicoletta Formenti
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
Flavia Guarneri
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini
Paolo Pasquali
Istituto Superiore Di Sanita
Giovanni Loris Alborali
Istituto Zooprofilattico Sperimentale della Lombardia e dell Emilia-Romagna Bruno Ubertini

Short communication

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Abstract

Background

The serovar Typhimurium (4,[5],12:i:1,2), is the most frequently isolated serovar in case of illness in pigs in Europe and its monophasic variant (4,[5],12:i:-) has been increasingly responsible for *Salmonella* outbreaks in humans. A total of 29,549 samples were collected, during the years 2002–2017, from 1,359 pig farms located in Northern Italy. Samples were collected from different material sources including fecal samples, gut content and different organs.

Results

Salmonella was isolated in 16.94% of samples and, among the isolates, 733 were typed as *Salmonella* Typhimurium (ST) or its monophasic variant (MST). Over time, there was a progressive increase of isolation of MST which outnumbered ST. Most of the strains were isolated in animals during the weaning stage and the growing – fattening period whereas the clinical cases were mainly present in young pigs after weaning.

Conclusion

This study confirms the role of pig as a source of ST and its monophasic variant MST. In the last few years, ST has been progressively replaced by MST suggesting that MST has a competitive advantage over ST, probably due to its reduced virulence which renders the infection stealthier to recognize and control.

Background

Pigs can be infected with a broad range of *Salmonella* serotypes some of which can cause clinical disease and, frequently, can contaminate meat products (1).

Apart from the serovar Choleraesuis of *S. enterica subsp. enterica*, a host-adapted serovar usually isolated in cases of septicemia, the serovar Typhimurium, is now the most frequently isolated serovar in case of illness in pigs in Europe (2–4) and in the United States (5).

Clinically ill pigs can develop, in the most severe cases, enterocolitis and exhibit diarrhea and dehydration. The disease most commonly develops in pigs with concurrent debilitating diseases, in conditions of poor hygiene that allow exposure to high doses of the organism, or in immunologically naive pigs. Mortality is variable. Most pigs have a complete recovery and eliminate the organism, but others may remain carriers and intermittent shedders for several months (1).

The monophasic variant of *Salmonella* Typhimurium (4,[5],12:i:-) has been increasingly responsible for *Salmonella* outbreaks in humans, being the third most commonly reported in the EU in 2012 (6), and frequently reported across the world (7–9). This serovar was rarely identified before the mid-1990s but its isolation in both animals and humans has increased in the last 20 years (4, 10–12).

Salmonella prevalence varies widely among farms and at different growth stages within the same farm and, due to the high number of variables and the complex relationships among pathogen and host, definitive understanding of the transmission, shedding and carrier states of salmonellae are still difficult (1).

The aim of this study was to describe and evaluate the comparative occurrence of *Salmonella* Typhimurium (ST) and its monophasic variant *Salmonella enterica subsp. enterica* 4,[5],12:i:- (MST) in pigs and its association with clinical conditions.

Materials And Methods

Strains isolation

A total of 29,549 samples were collected, during the years 2002–2017, from 1,359 pig farms located in Northern Italy and regularly (at least once a year) checked for enteric pathogens. Samples were collected from different material sources including fecal samples, gut content and different organs (in particular spleen, liver and lymph nodes) and sent to the lab by farm vets.

The isolation and identification of *Salmonella* isolates were carried out always by the same lab, initially in accordance with ISO 6579:2002 and later, for samples collected since 2007, in accordance with ISO 6579:2007 amendment 1. Briefly, the samples were pre-enriched with Buffered Peptone Water (BPW) and incubated at 37 °C ± 1 °C for 18 ± 2 hours. The samples were then transferred, for enrichment, to Rappaport – Vassiliadis Soya Broth (RVS), incubated at 41.5 °C ± 1 °C for 24 ± 3 hours, and Mueller – Kauffmann Tetrathionate with Novobiocin Broth (MKTTn), incubated at 37 °C ± 1 °C for 24 ± 3 hours (samples 2002–2007), or to a Modified Semisolid Rappaport – Vassiliadis (MSRV) agar medium incubated at 41.5 °C ± 1 °C for 24 ± 3 hours (samples 2007–2017). MSRV agar plates were incubated for further 24 ± 3 hours if negative. Enrichment cultures were used to inoculate two solid media incubated at 37 °C ± 1 °C for 24 ± 3 hours: Xylose Lysine Deoxycholate agar (XLD) and Brilliant Green Agar (BGA).

Colonies of presumptive *Salmonella* were subcultured on Triple Sugar Iron (TSI) agar at 37 °C ± 1 °C for 18 ± 2 hours and further identified biochemically and confirmed as *Salmonella* by slide agglutination using a polyvalent O antiserum.

Strain serotyping

The complete serological characterization of *Salmonella* was performed by slide agglutination for the determination of somatic antigens, while, for the determination of flagellar antigens, the method of tube agglutination was followed according to the Spicer (13) technique, modified by Morris et al. (14).

In particular, the characterization of monophasic variant of *Salmonella* Typhimurium, was performed through two consecutive phase inversions by passaging through a U-shaped glass tube containing semisolid agar with H:i antiserum. Those isolates that still did not display the second phase after the first and the second passage were considered, phenotypically, monophasic.

Phage-typing

Phage-typing was performed at the Italian National Reference Centre for Animal Salmonellosis according to Anderson et al. (15). Typing of MST strains started in 2011 and for this reason only 225 isolates were typed. The total number of typed ST strains was 235.

Clinical case definition

We considered a “clinical case” (C) as an illness of variable severity commonly manifested by diarrhea with presence of *Salmonella* and in absence of isolation of other enteric pathogens. When *Salmonella* isolation occurred and no enteric signs were shown the condition was referred to as a “non-clinical case” (NC).

Statistical methods

Statistical analysis was performed using GraphPad 6.0 software for MAC OS X (GraphPad Software Inc.; San Diego; CA). Differences in proportions were estimated using Fisher’s exact test. A $P \leq 0.05$ was considered statistically significant.

Results

Serotyping results

Salmonella was isolated from 5,005 out of 29,549 fecal samples (16.94%). Among the isolates, 733 were typed as *Salmonella* Typhimurium (n = 246) or its monophasic variant (n = 487). 285 out of 1,359 farms (20.97% of the total) resulted positive for the serovars investigated in this study. The distribution of the isolates, during the considered period, is showed in Fig. 1. Overall, it is possible to see two distinct phases. The number of ST isolates were slightly greater than that of MST isolates from 2002 to 2007. Thereafter, since 2008, it is possible to see a steady increase of the presence of MST over ST.

517 out of 733 collected isolates came from pigs whose different ages were known: amongst them, most of the MST and ST strains were isolated in animals during the weaning (since 30 days of age till 25/30 kg weigh) stage (n = 311; 60.15%) and the growing (25/30 kg – 60 kg weigh) – fattening (60 kg weigh to slaughtering) period (n = 177; 34.24%) whereas the number of isolates from breeders (n = 11; 2.13%) as well as from suckling piglets (n = 18; 3.48%) was low. Similar patterns of distribution were observed considering ST and MST separately (Fig. 2).

Clinical signs were associated with 114 out of 246 (46.34%) isolates of ST and 184 out of 487 (37.78%) isolates of MST (Table 1). Although it seems that the association of MST to clinical signs is lower than in

ST, the difference only approached the statistical significance (P = 0.08).

Figure 3 shows the percentage of strains (on the total of MST and ST strains) associated with clinical illness referred to the single production stage. Most of the clinical cases were present in young pigs after weaning, while in fatteners and breeders the occurrence of clinical signs were lower. When infected with MST, clinical signs were present in 45.17% (103/228) and 25.95% (34/131) of pigs, in weaners and growers-fatteners respectively, while in case of isolation of ST, clinical signs were present in 53.01% (44/83) and 43.48% (20/46) of pigs, respectively.

Table 1

Association between clinical conditions and the presence of *Salmonella* Typhimurium (ST) and its monophasic variant (MST)

	Clinical signs present (number of isolates)	**Clinical signs absent (number of isolates)**	**Total number of isolates**
ST	114	132	246
MST	184	303	487
Total	**298**	**435**	**733**

Phage typing results

The isolates belonged to 23 different phage types (Table 2), and eight of them were common to both serovars. The most frequently isolated phage types were DT193 (62 isolates) and DT120 (47 isolates), followed by U311 (45 isolates) and U302 (29 isolates). Other types found were DT104 (18 isolates), DT20A (16 isolates) and DT208 (11 isolates). DT193 was the most frequent type among MST strains while U302 was the most common among ST strains. 49 isolates showed a pattern which did not conform (RDNC) to any defined pattern, and 149 could not be phage typed (NT).

Table 2

Phage-types associated with isolates of *Salmonella* Typhimurium (ST) and its monophasic variant (MST)

Phage type	MST	ST	TOTAL
DT193	50	12	62
DT120	36	11	47
U311	28	17	45
U302	4	25	29
DT104		18	18
DT20A	13	3	16
DT208		11	11
DT110	1	6	7
DT104B		5	5
DT12		5	5
DT7VAR		3	3
DT1	1	1	2
DT32	1	1	2
DT138		1	1
DT193A	1		1
DT194		1	1
DT195		1	1
DT27		1	1
DT36		1	1
DT7		1	1
DT7A	1		1
DT99		1	1
U310		1	1

Discussion

The results presented here were obtained from farms located in Northern Italy in a high-density pig population area and regularly (at least once a year) checked for enteric pathogens. We focused on ST and MST, considering the prominent role of these two serovars in the pig population (4, 12).

ST and MST represented 4.92% and 9.73%, respectively of the *Salmonella* serovars isolated. These figures are not consistent with recent reports suggesting that ST and MST represent between 40% and 50% of the Italian isolates, with MST increasing from 9.66–46.34% in the last ten years (16, 17). A similar increasing has also been reported in other countries (12).

The distribution of the studied serovars during the considered period highlighted a predominance of ST on MST in the first period and, since 2008, a reversion of this tendency with MST becoming more and more predominant over the years. These data are in accordance with recent reports where the increasing prevalence of MST is well documented, in particular in the United Kingdom, Poland and Malta (6, 18, 19). In particular, in the United Kingdom, MST represented 60.7% of the *Salmonella* isolates obtained from a surveillance program in pigs in 2015 (20).

It is conceivable to hypothesize that MST has a selective advantage over ST. It was suggested that a number of factors (i.e. involvement of prophages and antigenic changes) can cause a reduced immune response to MST in herds when compared to ST (4). More recently, a comparative whole-genome sequencing and phylogenomic analysis of MST isolates from the United Kingdom and Italy during the period 2005–2012, revealed a high level of microevolution that may affect antigenicity, pathogenicity, and transmission (21). We recently explored the ability to induce an immunity using an inactivated MST (22) or an attenuated variant of ST (23, 24), yet we found a limited cross reaction between MST and ST.

When considering the production stage, both ST and MST showed their highest presence in the weaning and growing period as reported previously (25, 26). A comparison between the prevalence of ST and MST in different production stages showed no significant differences. Overall, these findings suggest a higher level of susceptibility in younger pigs, irrespective of the serovars involved. Pigs can become infected at any production stage but the decline of maternal antibodies after weaning makes younger pigs more susceptible to the infection (27).

When considering the association between clinical signs and isolates, we observed that clinical signs were associated more to ST than to MST and that most of clinical cases were present in young pigs after weaning. These data, although only approaching significance, are supportive of a lower virulence of MST compared to ST. In addition, it is worth mentioning that data obtained in our laboratory seem to show that MST is less virulent than ST in either pig or mouse models of experimental infections (Alborali G.L. and Pasquali P., personal communication).

The phage-typing highlighted the prevalence of four types representing about 70% of the typed isolates (DT193, DT120, U311, U302) and this has been a common feature of European isolates for the last twenty years (11, 28, 29). DT193 has to be regarded as an important phage type also for ST, considering its increase in Europe in the last years (30) and its role in human cases of salmonellosis.

Conclusions

In conclusion, this study confirms the role of pig as a source of ST MST. In the last few years, ST has been progressively replaced by MST suggesting that MST has a competitive advantage over ST, probably due to its reduced virulence which renders the infection stealthier to recognize and control. Here, however, lower virulence has only been hypothesized and more detailed studies should be undertaken to confirm this and, more importantly, to assess the mechanisms underpinning the competitive advantage of MST over ST in pigs.

Abbreviations

ST
Salmonella Typhimurium
MST
Salmonella Typhimurium monophasic variant
EU
European Union
RDNC
result does not conform
NT
not typed
ISO
International Standard Organization
BPW
Buffered Peptone Water
RVS
Rappaport – Vassiliadis Soya
MKTTn
Mueller – Kauffmann Tetrathionate with Novobiocin
MSRV
Modified Semisolid Rappaport – Vassiliadis
XLD
Xylose Lysine Deoxycholate
BGA
Brilliant Green Agar
TSI
Triple Sugar Iron
C
clinical case
NC
non-clinical case

Declarations

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Authors' contributions

GLA, MD: experimental design

SG, JR, CS, FS, MT, NF, FG: collection and analysis of the samples

MD: text elaboration of the manuscript

MD, PP: discussion and interpretation of the results

GLA: supervision of the activities

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The dataset supporting the conclusions of this article is included within the article.

Ethics approval and consent to participate

All farms enrolled in the study followed their own management practices. No protocol approval of the ethical committee of Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna "Bruno Ubertini" was required as samples were collected at slaughterhouse after processing. Informed consent was received from all animal owners.

Competing interests

The authors declare that there are no potential conflicts of interest or any financial or personal relationships with other people or organizations that could inappropriately bias conduct and findings of this study.

Consent for publication

Not applicable.

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Figures

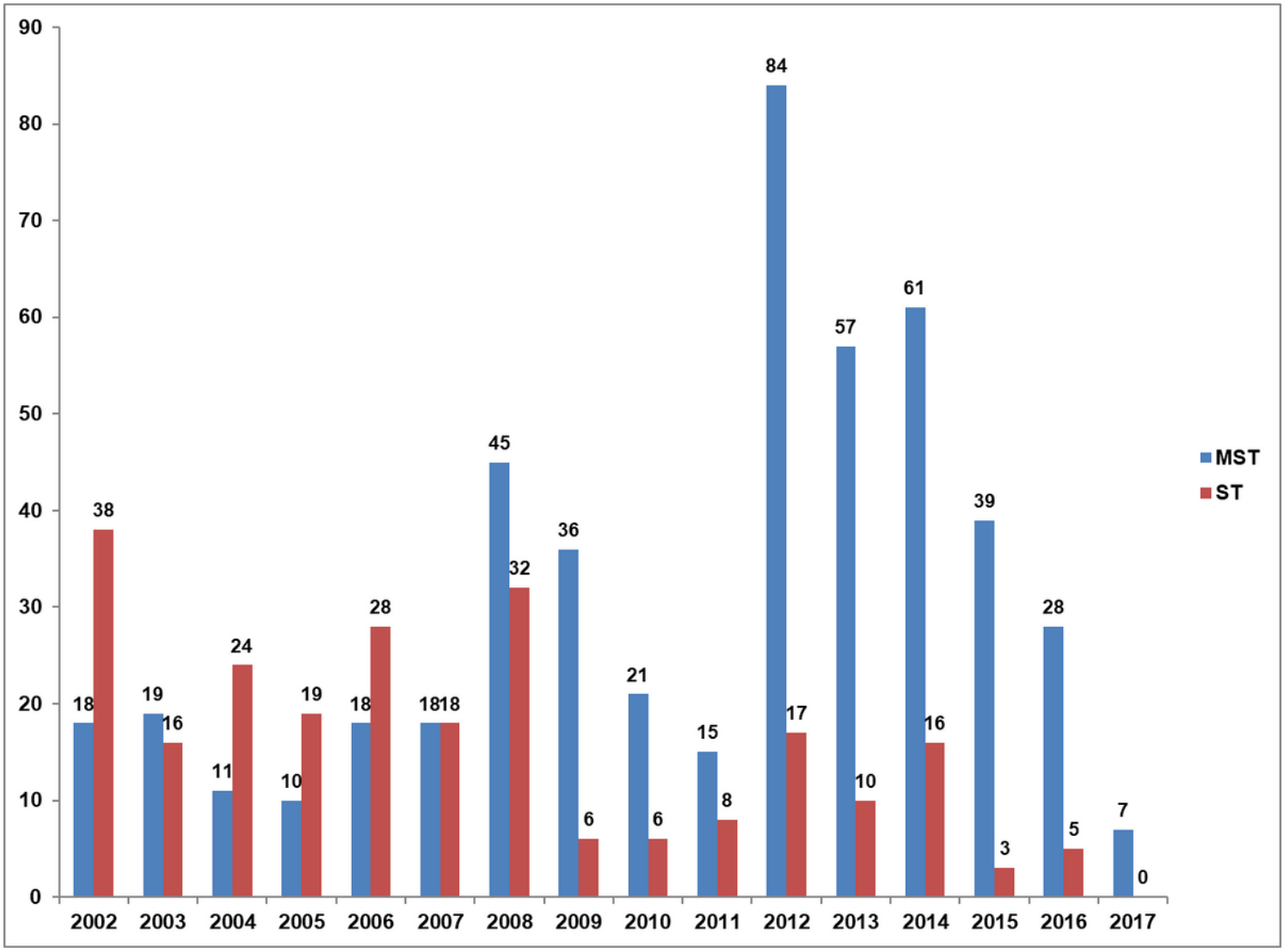
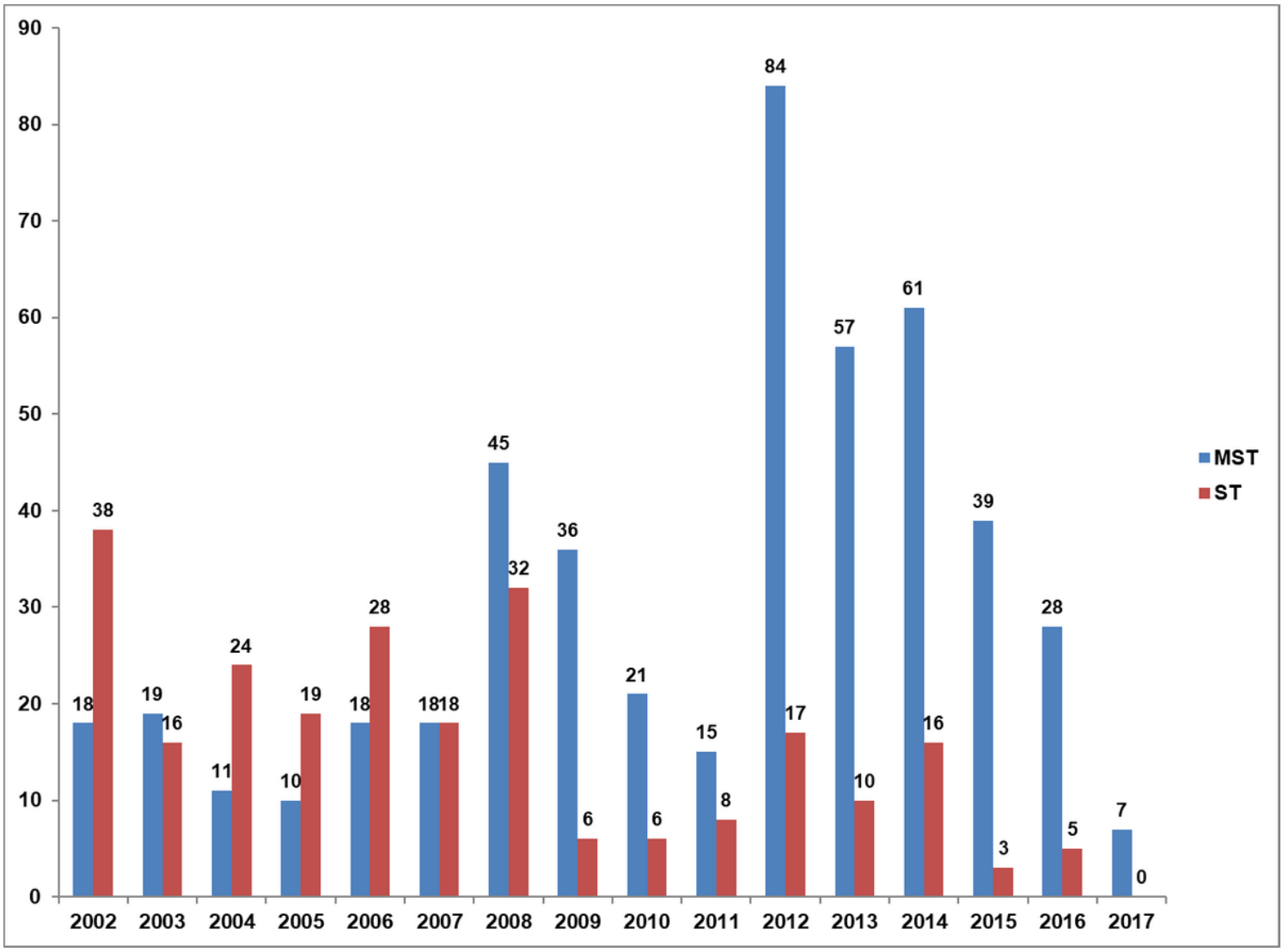


Figure 1

Distribution of the isolates of Salmonella Typhimurium (ST) and its monophasic variant (MST), during the period of the study (2002 – 2017)



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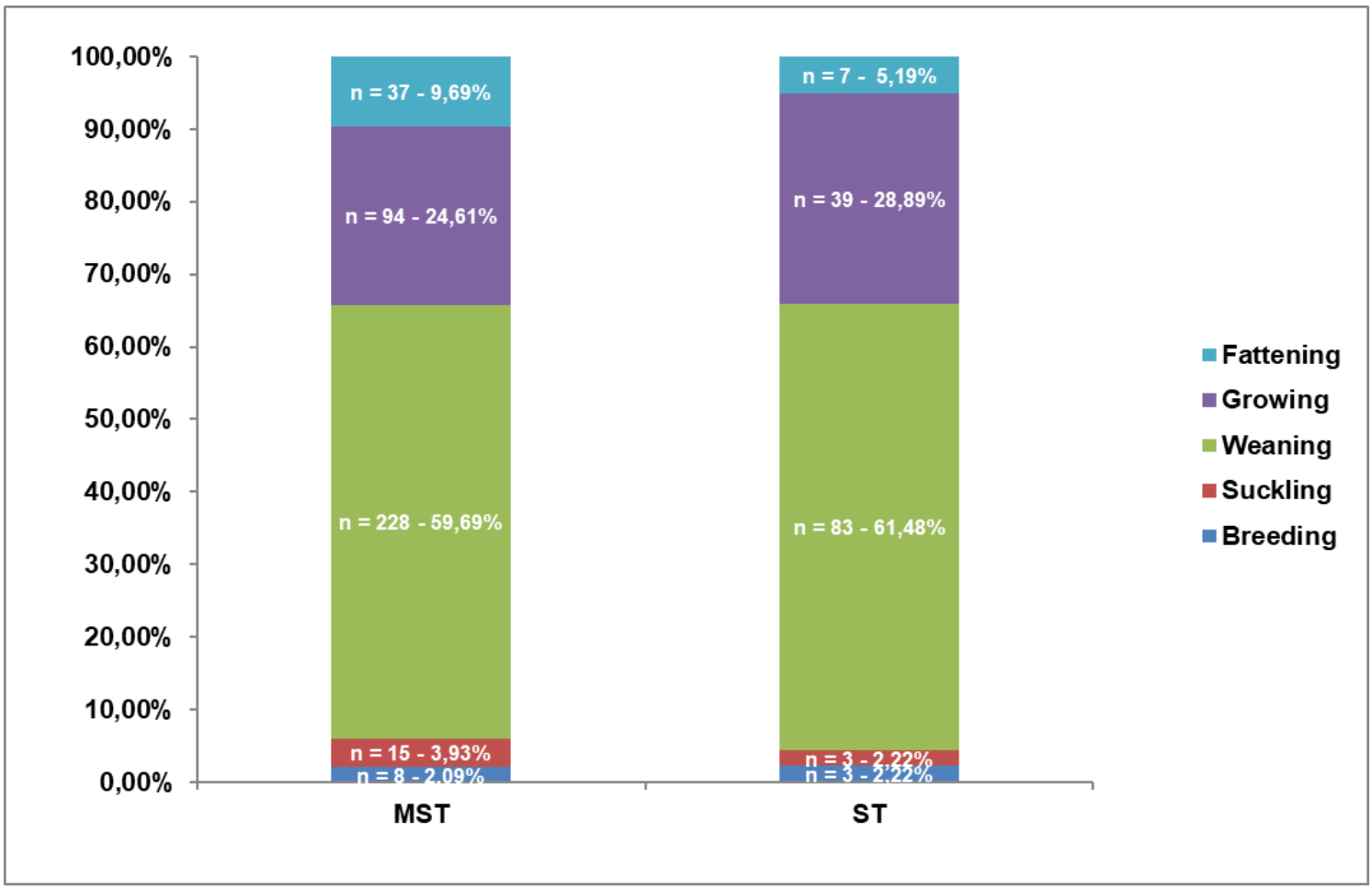
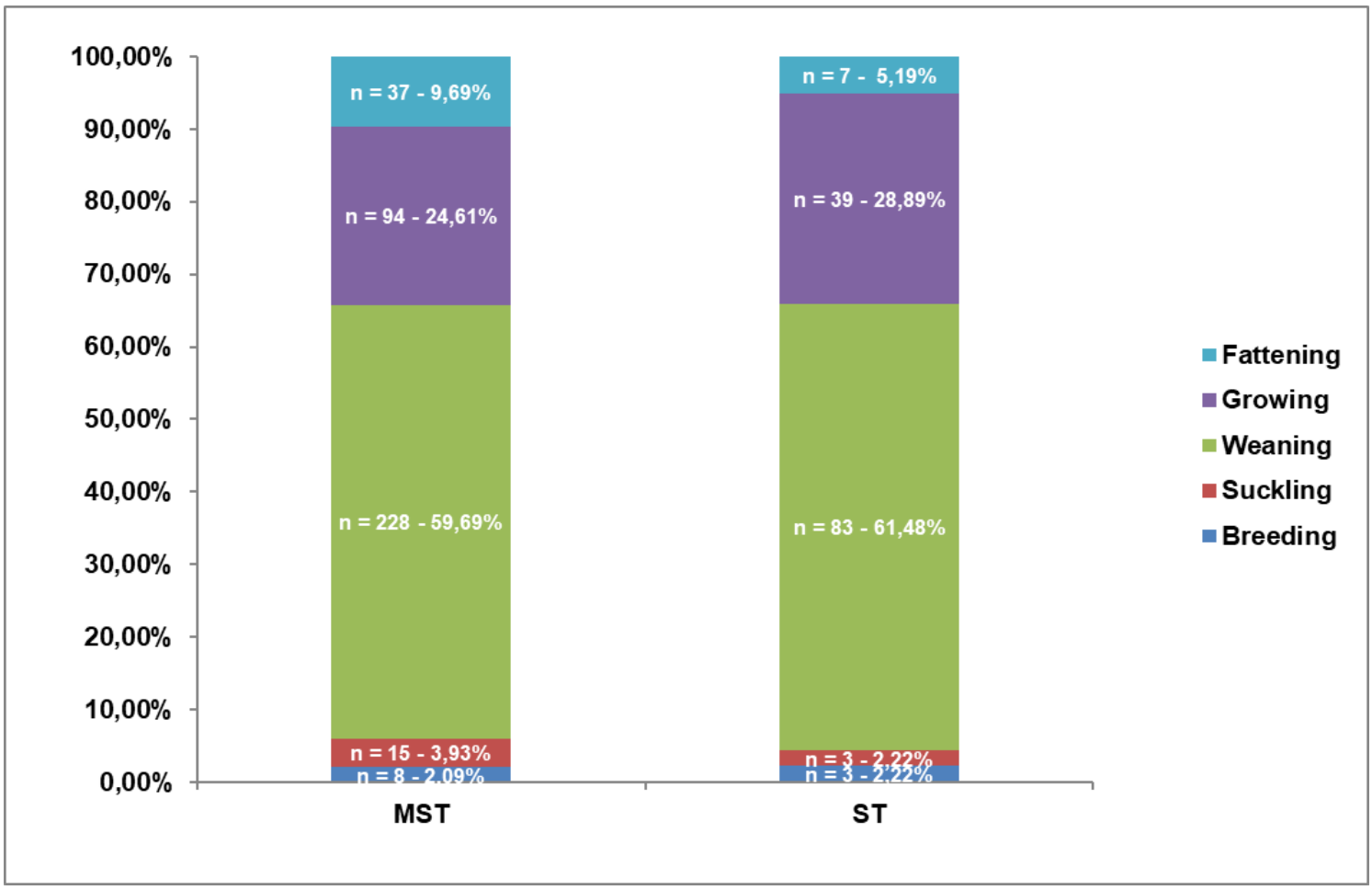


Figure 2

Distribution of the isolates of Salmonella Typhimurium (ST) and its monophasic variant (MST) related to the production stages



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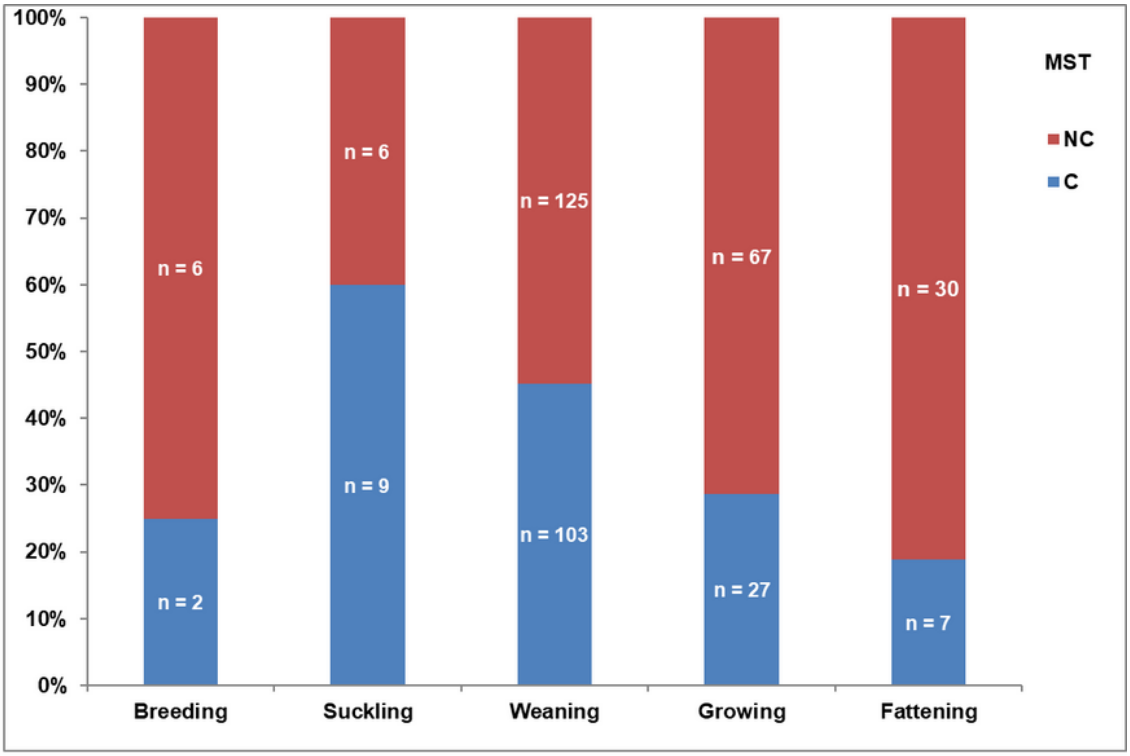


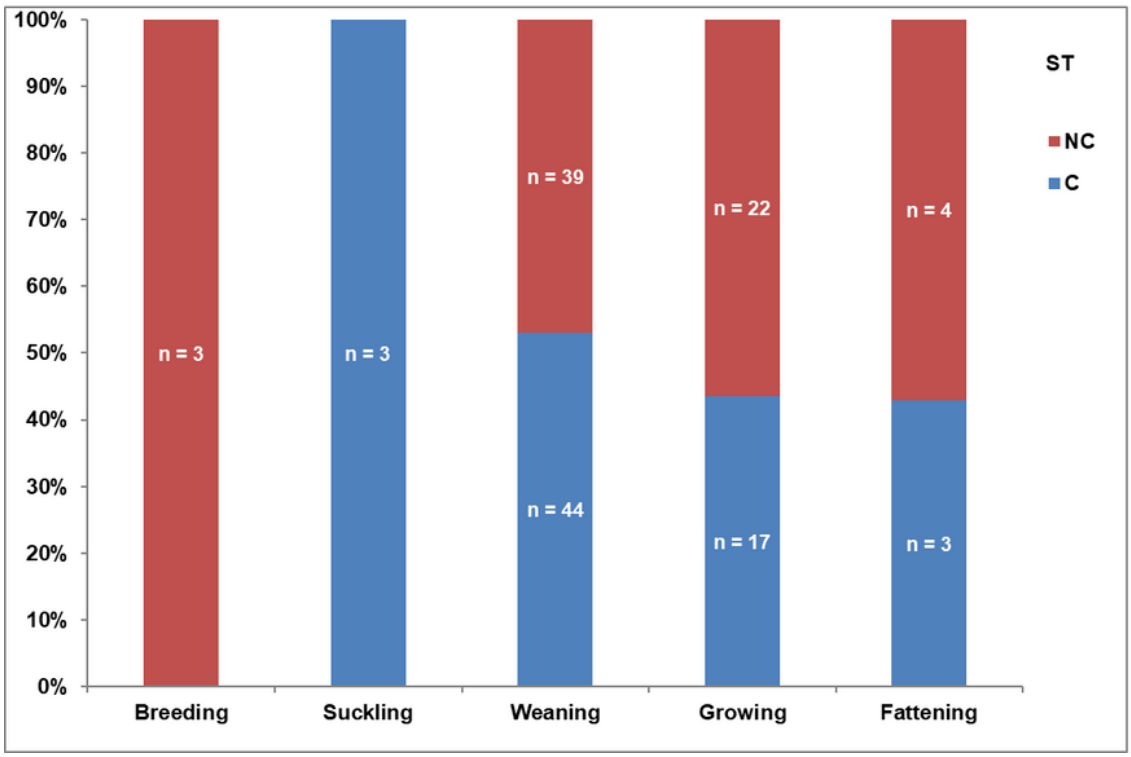
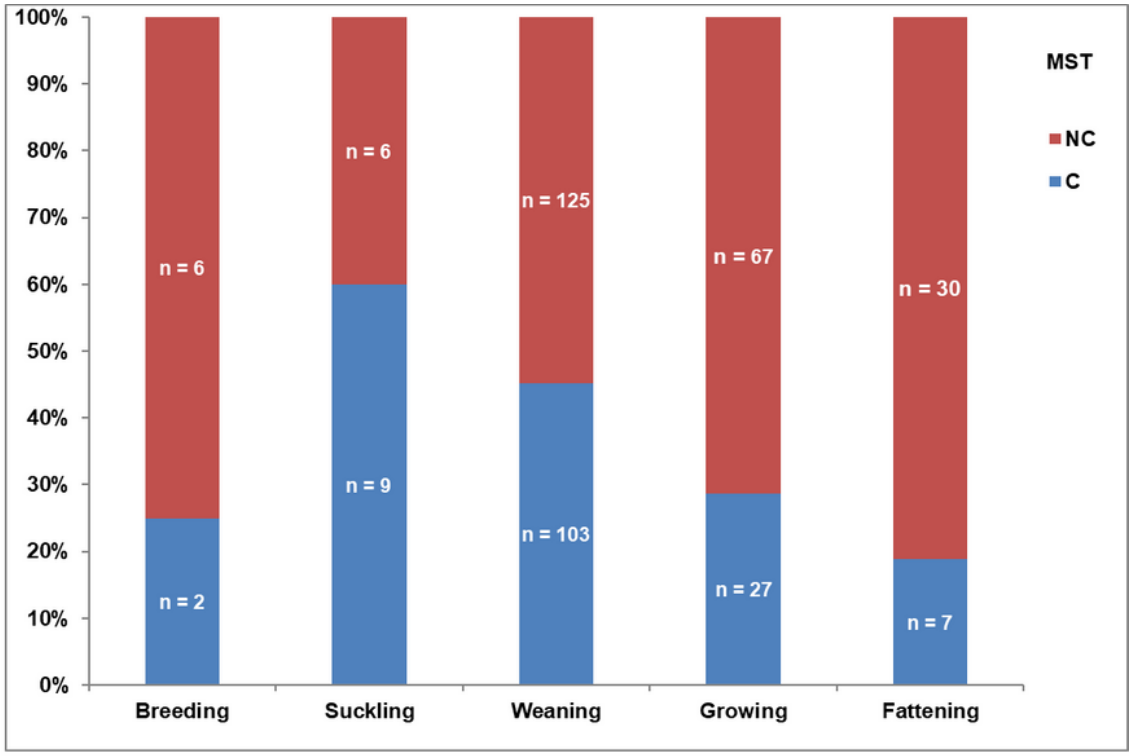
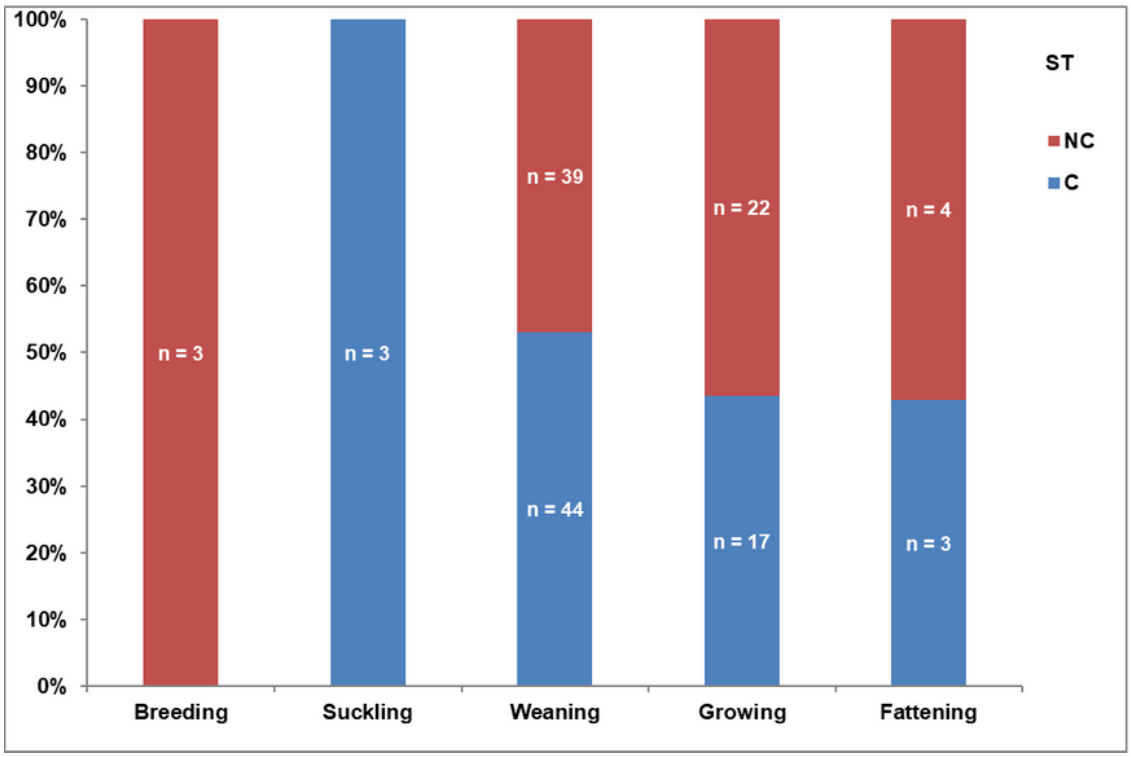


Figure 3

Percentage of the isolates of Salmonella Typhimurium (ST) and its monophasic variant (MST) associated (C) or not associated (NC) with clinical illness related to the production stages





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